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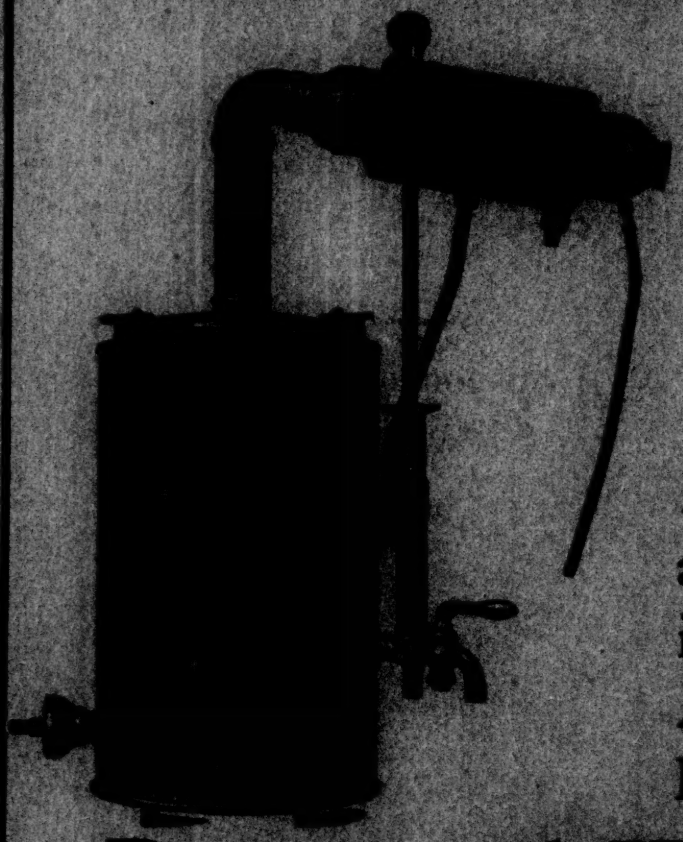

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IMPURE PICRIC ACID AS A SOURCE OF ERROR IN CREATINE AND CREATININE DETERMINATIONS.

By OTTO FOLIN and E. A. DOISY.

(From the Biochemical Laboratories of the Harvard Medical School and the Massachusetts General Hospital, Boston.)

(Received for publication, November 8, 1916.)

In the course of the last few months McCrudden and Sargent have published two critical papers on the colorimetric method for the determination of creatine and creatinine. Their first paper[1] describes some seemingly simple yet convincing experiments indicating that normal human urine contains some substance ("x-creatine") which gives the creatinine reaction when boiled with picric acid as in Folin's micro method[2] for the determination of creatine, yet gives no such reaction after heating with hydrochloric acid (as in the older macro method). The second paper[3] has to do with the influence of the color from the sodium picrate in the determination of creatinine in blood and urine. The last two paragraphs of this paper are as follows:

"From the data it is clear that in the determination of creatinine in the blood the color due to creatinine is such a small proportion of the total color that *analysis gives no information whatever concerning the amount of creatinine present;* the slight variations obtained in duplicates can be accounted for by slight variations in the amount of picric acid in the solution.

In the light of these experiments it is clear that all that has been written hitherto concerning the physiology of creatinine and creatine needs careful revision; much of it will have to be modified, some of it—all that concerning creatinine and creatine in the blood, for example—will have to be rejected altogether."

We have repeated the work of McCrudden and Sargent with reference to the determination of creatine in urine and of creati-

[1] McCrudden, F. H., and Sargent, C. S., *J. Biol. Chem.*, 1916, xxiv, 423.
[2] Folin, O., *J. Biol. Chem.*, 1914, xvii, 472, 479.
[3] McCrudden and Sargent, *J. Biol. Chem.*, 1916, xxvi, 527.

nine in blood, and in both instances have obtained results which are entirely different from the results reported by them.

Table I, containing some calculated and experimental figures taken from the second paper of McCrudden and Sargent and also the corresponding experimental figures obtained by ourselves, indicates the magnitude of the discrepancy between their data and ours.

TABLE I.

A Comparison of Calculated Colorimetric Readings with Those Obtained (a) by McCrudden and Sargent and (b) by Ourselves.

No.	a.	b.	Calculated.
	mm.	*mm.*	*mm.*
1	25.5	26.6	28.6
2	21.7	24.8	25.0
3	23.4	22.3	22.2
4	20.0	20.0	20.0
5	21.2	18.2	18.2
6	18.7	16.7	16.7
7	20.4	15.7	15.4
8	18.0	14.3	14.3

The differences between our results and those of McCrudden and Sargent manifestly lie far outside any reasonable limits of experimental error, and cannot be explained on the basis of some slight variation in the conditions under which the two sets of determinations were made. In the course of our endeavor to find an adequate explanation we examined the different samples of picric acid in our laboratory with reference to the color of their sodium picrates in terms of creatinine. McCrudden and Sargent had made such a comparison and had found that creatinine, weight for weight, gives about 500 times as deep a color with picric acid and alkali as is given by the picric acid and alkali alone. In all we examined six different samples of picric acid, of which four had been bought in dry form and the fifth in the more recent wet condition in which picric acid is now almost always sold; the sixth was a product obtained from the fifth by recrystallization of the sodium salt, according to directions given below.

According to our observations creatinine gave about 3,000 times as deep a color as was given by our poorest picric acid (the wet sample), when alkali was added exactly as in the creatinine determination. Using our best, the recrystallized picric acid, creatinine gave about 12,000 times as deep a color as its sodium picrate.

In view of the remarkably faint color of our sodium picrates in comparison with the color recorded by McCrudden and Sargent, it occurred to us that the endeavor of American manufacturers to meet the enormous demands, created by the war, for picric acid, may have resulted in the production of extremely low grades of picric acid, and that McCrudden and Sargent may have been using an unusually impure sample.

We therefore communicated with Dr. McCrudden, and, with him, tested his picric acid and compared it with ours. The results obtained were quite astounding, and it became at once clear to us, as well as to Dr. McCrudden, that the observations published by McCrudden and Sargent had been obtained on the basis of an extraordinarily impure picric acid. The results were so different from the results obtained with any sample of our picric acid that the conclusions formulated by McCrudden and Sargent have no bearing whatever on the results obtained with approximately pure picric acid.

It remains to be said, however, that the picric acid used by McCrudden and Sargent was labelled C.P., and it is scarcely to be supposed that other investigators may not have bought and used for creatinine determinations picric acid of similar low grade. We agree with McCrudden and Sargent that any creatinine or creatine determinations made on blood by the help of such impure picric acid would be worthless.

From our examination of a number of different samples of wet picric acid we have obtained the impression that with the introduction of this "safe" mode of shipping, the manufacturers must have introduced some unsatisfactory change in the process of preparing the product. We have not yet seen a single sample of wet picric acid as pure as any of the four dry samples in our possession.

As a simple test of the purity of picric acid for use in connection with creatinine determinations we propose the following.

To 20 cc. of a saturated (1.2 per cent) solution of picric acid add 1 cc. of 10 per cent sodium hydroxide and let it stand for 15 minutes. The color of the alkaline picrate solution thus obtained must not be more than about twice as deep as the color of the saturated acid solution. If the quality of the picric acid is good, the color of the picrate solution will be no deeper at the end of 24 hours than at the end of 15 minutes provided that organic impurities, dust, etc., be excluded. If the picric acid is unusually pure, the color of the picrate solution will not be more than one and a half times as deep as that of a saturated picric acid solution; *i.e.*, by setting the picric acid solution at 20 mm. in the Duboscq colorimeter, the picrate will give a reading of 13 to 14 mm.

To purify picric acid proceed as follows: Transfer about 600 gm. of wet picric acid, or about a pound of dry picric acid, to a large beaker (capacity not less than 4 liters). Pour on boiling water until the beaker is nearly full and add 200 cc. of saturated (50 per cent) sodium hydroxide solution. Stir, and if necessary heat again until all the picric acid has dissolved, yielding a deep red picrate solution. To the hot solution add rather slowly, with stirring, 200 gm. of sodium chloride. Cool in running water to about 30°C., with occasional stirring. Filter on a large Buchner funnel and wash a few times with 5 per cent sodium chloride solution. Transfer the picrate to the large beaker, fill with boiling water, and when the picrate is dissolved add, with stirring, first 50 cc. of 10 per cent sodium hydroxide solution, and then 100 gm. of sodium chloride. Cool to 30°C., with stirring, filter, and wash with sodium chloride solution, as before. Repeat the solution and precipitation of the sodium picrate twice more, but for the last washing of the last precipitated picrate use distilled water instead of sodium chloride solution.

Dissolve the purified picrate in the same large beaker, with boiling distilled water, and filter hot on a large folded filter, collecting the filtrate in a large flask. To the hot filtrate add 100 cc. of concentrated sulfuric acid, previously diluted with about two volumes of water. The liberated picric acid begins to come out at once. Put a beaker over the mouth of the flask and cool under running tap water to about 30°C. Filter with suction as before and wash free from sulfates with distilled water.

While the findings of McCrudden and Sargent are abundantly accounted for on the basis of impurities in their picric acid, it of course remains a fact that the yellow color of pure sodium picrate does theoretically interfere with the accuracy of the colorimetric creatinine estimations and practically does limit the dilu-

tion in which creatinine may be quantitatively determined. The creatinine solution obtained from normal blood after the proteins have been precipitated by the addition of four volumes of picric acid solution is strong enough in preformed creatinine to permit its estimation with a fair degree of accuracy.

A simple modification of the original procedure, which most investigators will probably accept as an improvement, is the following.

To the fresh blood add four volumes of saturated picric acid solution and about 1 gm. of powdered picric acid for each 10 cc. of blood used. Shake for 10 minutes, and filter. To 10 (or 20) cc. of the filtrate add 1 (or 2) cc. of a solution containing 7 per cent potassium hydroxide and 25 per cent potassium chloride. Let stand for 10 minutes, centrifuge, and compare in the colorimeter with the standard creatinine in picric acid to which has been added a corresponding amount of alkaline potassium solution. The potassium in the alkali precipitates fully 75 per cent of the picric acid present in a saturated solution, and thereby makes the color due to the minute amounts of creatinine present in normal blood distinctly more predominant than when the filtrates remain saturated solutions of picric acid.

In the above modification, as well as in the original method, the blood is diluted fivefold by the addition of four volumes of picric acid solution. The chief reason for this great dilution of the blood filtrates is that it enables one to make a creatinine determination with as little as 2 cc. of blood. When there is no reason for economy of blood, the creatinine concentration of the blood filtrates can advantageously be increased by using only two volumes of picric acid solution (+ solid picric acid) for the precipitation of the proteins. The precipitation in this case requires more time, about an hour. The standard creatinine solution in this case should, of course, be less dilute than when four volumes of picric acid are used for the precipitation.

Dr. McCrudden agreed with us that since we had been unable to find "x-creatine" in urine, this finding also is probably to be explained on the basis of impurities in the picric acid used by McCrudden and Sargent. To prove the point we determined the creatinine-creatine in three samples of urine, (*a*) by the old

method, (*b*) by the new (picric acid) method using our picric acid, and (*c*) by the new method using McCrudden's picric acid. The following values (in gm. per liter) were obtained.

TABLE II.

No.	a.	b.	c.
	gm.	*gm.*	*gm.*
1	1.63	1.64	2.09
2	1.33	1.35	1.67
3	1.71	1.73	2.23

The figures recorded in Table II prove conclusively that the highly impure picric acid used by McCrudden and Sargent was responsible for their finding creatine in urines which did not contain any creatine.

Before communicating with Dr. McCrudden we had made a long series of determinations of creatinine and creatine in human urine. In this series we had determined the creatine-creatinine both by the old process and by Folin's micro method. The figures obtained are not without interest, because (1) they prove that the two methods give identical results, and (2) they are numerous enough to give a good idea as to the frequency with which creatine occurs in human urine, in health and in disease. The figures are given in the form of colorimetric readings only.

In explanation of these figures (Table III) it should be stated that in determining the creatine-creatinine we used as the standard the creatinine of the unheated urine (and not a standard creatinine solution).

TABLE III.

No.	Subject.	Diet.	Colorimetric readings.		
			Creatinine 1 mg. standard set at 20 mm.	Creatine-creatinine. Creatinine as standard set at 20 mm.	
				New method.	Old method.
		Normal Men.			
1	Normal.....	Mixed.		17.3	17.4
2	"	"		18.5	18.5
3	"	Creatine-free.	16.6	20.0	20.1
4	"	"	13.2	20.0	20.1
5	"	"	12.3	20.6	19.8
6	"	"	19.3	20.1	20.1
7	"	"	7.0	20.1	20.1
8	"	"	12.9	20.1	20.0
9	"	"	15.5	19.8	19.9
10	"	"	22.1	20.0	19.9
11	"	"	14.1	20.0	19.9
12	"	"	25.7	20.1	20.2
13	"	"	20.0	20.2	19.9
14	"	"	21.3	20.2	20.1
15	"	"	19.1	20.2	20.1
16	"	"	16.8	19.8	20.1
17	"	"	17.8	19.9	20.1
18	"	"	12.1	20.1	19.9
19	"	"	14.9	19.9	20.2
20	"	"	12.0	20.0	20.2
21	"	"	12.5	20.0	20.2
22	"	"	19.7	19.9	20.5
23	"	"	12.7	18.9	18.7
24	"	"	8.6	18.2	18.1
25	"	"	13.2	20.0	19.9
26	"	"	12.7	19.8	20.1
27	"	"	21.4	20.0	20.2
		Pathological Men.			
28	Diarrhea.....	Creatine-free.	16.6	18.3	18.1
29	Nephritis.....	"	15.5	17.0	16.9
30	Leukemia.....	Mixed.	16.2	14.7	14.9
31	Cardiac.....	"	26.9	10.8	11.0
32	"	"	13.0	18.0	18.0
33	Valvular heart.....	"	15.0	17.9	17.7
34	Mitral stenosis.....	"	24.8	16.7	17.1
35	Lung process.....	"	22.8	17.9	17.7
36		"	18.2	18.3	18.3

TABLE III—*Concluded.*

No.	Subject.	Diet.	Colorimetric readings.		
			Creatinine 1 mg. standard set at 20 mm.	Creatine-creatinine. Creatinine as standard set at 20 mm.	
				New method.	Old method.
		Pathological Men.			
37	Tabes	Mixed.	15.1	18.7	19.1
38	Cirrhosis of liver	"	12.0	17.9	18.3
39	Renal anasarca	"	16.1	18.8	18.9
40	Pleurisy	"	15.0	16.8	16.6
41	"	"	19.1	17.8	18.0
42	"	"	17.8	17.6	17.8
43	"	"	13.3	17.2	17.2
44	"	"	16.2	14.5	14.8
45	"	"	19.6	8.8	5.2*
		Pathological Women.			
46	Enteric	Mixed.	12.5	15.8	15.7
47	"	"	16.3	17.4	17.4
48	"	"	14.0	14.4	14.4
49	Gastric ulcer	Creatine-free.	17.6	14.8	15.1
50	Pneumonia	"	27.9	16.6	16.6
51	"	"	14.5	12.0	12.2
52	"	"	19.0	11.0	10.3
53	"	"	17.3	9.9	10.1
54	"	"	18.0	16.1	16.0
55	"	"	15.8	17.2	17.2
56	"	"	17.6	16.0	16.1
		Pathological Children.			
57	Surgical	Mixed.	22.5	10.7	10.7
58	"	"	27.0	10.2	9.8
59	"	"	27.6	12.3	12.2
60	Syphilis	"	19.6	16.9	16.6
61	"	"	18.6	13.9	14.2
62	Eczema	Creatine-free.	20.4	14.1	14.2
63	"	"	16.4	10.6	10.4
64	"	"	14.5	17.9	18.2
65	"	"	27.1	17.1	17.1

* Sugar.

THE ESTIMATION OF AMINO-ACID NITROGEN IN BLOOD.

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(Received for publication, November 3, 1916.)

In spite of the rapidly growing literature upon the occurrence of amino-acids in blood and tissue under various conditions, little attention appears to have been given to a study of the comparative merits of various procedures for the preliminary removal of protein and manipulation of the solution prior to the analysis.

For the most part the original method of Van Slyke and Meyer[1] has been followed, in which the blood proteins are removed by precipitation with alcohol, the filtrate evaporated *in vacuo* to a small volume, and this solution used for analysis by the nitrous acid method.[2] Recent investigations by Folin and Denis[3] and particularly by Greenwald[4] have indicated that amino-acids do not completely escape precipitation by alcohol.

It was therefore considered desirable, as preliminary to some contemplated work upon the physiological occurrence of amino-acids, to compare the results obtained by the Van Slyke method, where different procedures were employed for the removal of the protein. It is the purpose of the present paper to report the results obtained in this connection, together with certain other findings which may be of interest.

Folin and Denis[3] state that certain nitrogenous substances added to the blood cannot be recovered quantitatively after precipitation with methyl alcohol.

Greenwald,[4] as a result of his study of this question, concluded that alcohol precipitates some nitrogenous non-protein con-

[1] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.
[2] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.
[3] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.
[4] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

stituents of the blood, of which 25 to 50 per cent represents amino-acid nitrogen, as determined by Van Slyke's method. As it was originally planned in the present writer's work to substitute the Greenwald procedure of blood precipitation with trichloroacetic acid for the alcohol precipitation, a somewhat detailed study of the two methods was made.

TABLE I.

Comparison between Alcohol Precipitation and Trichloroacetic Acid Precipitation of Blood. Non-Protein Nitrogen per 100 Cc. of Blood.

Material.	Methyl alcohol precipitation.	Trichloroacetic acid precipitation.	Remarks.
	mg.	*mg.*	
Sheep blood (oxalated).....	28.70	37.60	By micro Kjeldahl.
" " "	25.40	34.70	Distillates Nesslerized.
Ox " (defibrinated).....	23.25	26.25	
" " "	22.12	25.90	
" " "	22.02	23.30	
" " "	18.31	24.30	
Calf " (oxalated).....	26.30	29.55	
Ox " (defibrinated).....	21.10	25.60	
Ox blood (defibrinated).....	27.23	30.02	By micro Kjeldahl.
" " "	30.57	31.82	Distillates titrated.
Sheep " "	28.20	27.37	
" " "	28.29	30.01	
Ox " + amino-acids.....	35.57	39.77	
" " (defibrinated).....	28.59	28.40	
Sheep " (oxalated).....	34.98	39.53	
" " "	35.64	36.75	
Pig blood (oxalated).....	22.93	23.88	By Kjeldahl.
" " "	29.92	32.03	
" " "	28.15	30.81	
Sheep " "	35.76	39.71	
" " "	35.48	38.40	

Determinations of the total non-protein nitrogen were made first. Samples of blood were precipitated according to Folin and Denis[3] and Greenwald.[4] Aliquot parts of the filtrates were digested and distilled as described in a previous paper.[5] The results are summarized in Table I. The figures given for Nessleri-

[5] Bock, J. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 47.

zation represent equivalents of 1 cc. of blood, those for micro distillation and titration with methyl red as indicator represent 3 to 5 cc. of blood. The last part of Table I gives Kjeldahl determinations on amounts of filtrates representing 30 to 40 cc. of blood. The micro determinations represent averages of three to seven determinations. The Kjeldahl figures represent averages of two to three determinations each.

The results show a decided loss of non-protein nitrogen where alcohol was used as precipitant. The differences are largest where Nesslerization was employed. This may be due, at least in part, to the small quantity of blood used in these determinations, whereby the limit of error is correspondingly increased. This may explain the much greater differences obtained by Greenwald when compared with the results obtained by titration in the present writer's results.

The precipitation of blood for the determination of amino-acid nitrogen was then tried. As urea and ammonia react somewhat with nitrous acid in the Van Slyke procedure, it was necessary to remove these constituents beforehand. A measured volume of blood (30 to 50 cc.) was introduced into a flask which contained 0.3 gm. of ground soy bean, a little water (2 to 3 cc.), and 1 cc. of a 3 per cent solution of NaH_2PO_4, and the mixture was gently agitated. After standing for $\frac{1}{2}$ hour at room temperature, the mixture was precipitated by diluting to ten times its original volume with a 2.5 per cent solution of trichloroacetic acid. After standing for 30 minutes, it was filtered, the filtrate shaken with kaolin, and filtered again. An aliquot part of the filtrate was taken. The difficulty which presented itself at this stage was the removal of the trichloroacetic acid. The neutralization before removal is not practical, because the filtrate has to be evaporated to a very small volume and even fairly small amounts of salts give considerable trouble. The vacuum distillation, as in the original Van Slyke and Meyer method, was tried. The filtrate was evaporated at reduced pressure in a water bath. The usual arrangement of two distilling flasks was used and the reduced pressure obtained by means of a good water pump. The filtrate was evaporated nearly to dryness, 100 cc. of water were added, and the solution was again evaporated under reduced pressure. The residue was made alkaline with

1.0 N potassium hydroxide and the ammonia distilled off. The residue, after being slightly acidified with acetic acid, was transferred to a small glass evaporating dish and evaporated on a water bath. The amino-acid nitrogen was then determined in the micro apparatus as described by Van Slyke. The apparatus was exactly the same as described by that author,[6] except that the gas burette was smaller, holding only 3 cc.[7] Table II shows the results obtained by this procedure. It will be noted that the results by the vacuum evaporation are very low. A second

TABLE II.

Comparison of Direct and Vacuum Evaporation of Blood Filtrates Obtained by Trichloroacetic Acid Precipitation. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Vacuum evaporation.	Direct evaporation.
	mg.	*mg.*
Ox blood (defibrinated)	1.08	6.20
Sheep " (oxalated)	1.95	7.04
Ox " (defibrinated)	1.85	6.92
" " "	2.40	6.16
" " "	3.50	6.92
" " "	3.57	6.09
Sheep " "	2.74	6.89
Ox " + amino-acids	4.31	12.59
" " "	3.25	12.71
" " (defibrinated)	1.69	6.43

method of removing the trichloroacetic acid was therefore tried. The original filtrate was put into a flask, a drop of alizarin indicator was added and the liquid brought to boiling. It was then kept boiling very slowly for 20 to 45 minutes until the indicator showed that the trichloroacetic acid had been removed. Enough 1.0 N potassium hydroxide was added to make the liquid distinctly alkaline and the ammonia removed by boiling from 1 to 2 minutes. It was then slightly acidified with acetic acid, boiled down further, and quantitatively transferred to a small evaporating dish. After evaporating to a small volume, the

[6] Van Slyke, *J. Biol. Chem.*, 1913–14, xvi, 121.
[7] Van Slyke, *J. Biol. Chem.*, 1915, xxiii, 407.

amino-acid nitrogen was determined. Table II compares the results obtained by the vacuum evaporation and the direct evaporation method.

The low figures by the vacuum distillation procedure required further study. The two processes were therefore reversed; *i.e.*, after evaporating *in vacuo* and before making alkaline, the residue in the distilling flask was transferred to a Florence flask, 2.5 per cent trichloroacetic acid was added to approximately the original volume, and the direct evaporation procedure applied. The residue from the direct evaporation was in turn evaporated *in vacuo* after making to volume with 2.5 per cent

TABLE III.

Vacuum evaporation.	Vacuum evaporation followed by direct evaporation.	Direct evaporation.	Direct evaporation followed by vacuum evaporation.
*A. Amino-Acid Nitrogen per 100 Cc. of Blood.**			
mg.	*mg.*	*mg.*	*mg.*
1.79	6.33	7.13	1.91
0.71	6.92	7.08	
3.15	7.22	7.24	
2.17	7.12	6.15	3.60
2.63	7.11	6.17	3.40
B. On Pure Alanine Solution (Calculated 1.2 Mg. of Amino-Acid Nitrogen)			
0.63	1.14	1.16	
0.66	1.18	1.22	

* Different samples of ox blood were used.

trichloroacetic acid. In the case of vacuum evaporation the results were invariably lower in the same ratio as obtained before. Table III summarizes the results. Each series was run on the same filtrates, obtained by precipitating large amounts of blood with trichloroacetic acid as described above.

Solutions of pure alanine were also tried under the same conditions. To 5 cc. of a solution of pure alanine 200 cc. of 2.5 per cent trichloroacetic acid were added and then treated as before; *i.e.*, (*a*) vacuum evaporation, (*b*) direct evaporation, and (*c*) vacuum evaporation followed by direct evaporation with 200

cc. of 2.5 per cent trichloroacetic acid. Table III B shows these results.

The differences, although not so pronounced as in the blood filtrates, show that the vacuum evaporation gives consistently lower results. This is apparently due to the fact that the trichloroacetic acid is not entirely removed by the vacuum evaporation and enters into some combination with the amino-acids which prevents their quantitative reaction in the Van Slyke method. If pure alanine solutions, after being concentrated to a small volume, are treated with solid trichloroacetic acid and allowed to stand at 40–80°C., they invariably give figures for amino-acid nitrogen which are from 10 to 30 per cent lower than the theoretical. The same observation was made on blood filtrates when treated with solid trichloroacetic acid after free evaporation.

A comparison between the methyl alcohol precipitation method and the trichloroacetic acid procedure was then made. 30 to 50 cc. of blood were allowed to stand with urease for ½ hour, then precipitated with nine times the volume of alcohol. After standing for 2 hours the precipitate was filtered off by suction, the filtrate treated with a little alcoholic $ZnCl_2$ solution, and filtered again. An aliquot portion of the filtrate was evaporated on a water bath to drive off the alcohol, and a little water was added. The liquid was made alkaline with 1.0 N KOH and boiled to drive off the ammonia. It was then acidified with acetic acid, evaporated, and the amino-acid nitrogen determined.

The precipitation with trichloroacetic acid was carried out as described before. Table IV compares the results obtained by the two methods on different bloods. The recovery of amino-acids added to the blood by the two procedures was also investigated. The amino-acid solutions used were obtained by hydrolyzing pure casein with strong HCl as described by Fischer.[8] After removing the HCl as far as possible by vacuum distillation, the solution was diluted to a convenient concentration of amino-acid nitrogen and treated by the Van Slyke method. As ammonia is formed in the hydrolysis of the casein, it is necessary to remove this before the determination of the amino-acid nitrogen is made.

[8] Fischer, E., Untersuchungen über Aminosäuren, Polypeptide und Proteine, Berlin, 1906, 55.

TABLE IV.

Comparison of Methyl Alcohol and Trichloroacetic Acid as Blood Precipitants for the Determination of Amino-Acids. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Methyl alcohol.	Trichloroacetic acid.
	mg.	*mg.*
Ox blood (defibrinated)	7.79	8.91
" " "	7.14	7.09
" " "	5.74	6.16
" " "	6.54	6.66
Sheep " "	6.86	6.89
" " "	6.09	8.19
Calf " (oxalated)	5.17	6.66
Ox " + amino-acids	10.44	12.59
" " (oxalated)	5.13	6.89
" " (defibrinated)	5.07	6.43
" " + amino-acids	9.53	12.71
Sheep " (oxalated)	3.72	7.82
" " "	4.25	7.79

TABLE V.

Recovery of Amino-Acids Added to the Blood by Methyl Alcohol and by Trichloroacetic Acid Precipitation. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Methyl alcohol.				Trichloroacetic acid.			
	Blood.	Blood + amino-acid.	Difference.	Calculated.	Blood.	Blood + amino-acid.	Difference.	Calculated.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Ox blood	5.13	10.44	5.31	6.28	6.89	12.59	5.70	6.28
" "	5.07	9.53	4.46	6.28	6.43	12.71	6.24	6.28

The results obtained by the methyl alcohol precipitation method are invariably lower, and the recovery of added amino-acids is not so complete as in the case of the trichloroacetic acid procedure (Greenwald[4]).

The latter procedure gives satisfactory results, but is somewhat troublesome in certain stages of the manipulation. Filtration is very slow, and the alternative process of centrifuging such large

volumes (300 to 500 cc.) is not always convenient. Another method was therefore sought.

The main difficulty with most precipitation methods is that the filtrates contain too large an amount of solids in solution to permit evaporation to the small volume necessary for the final determination. Therefore a procedure was adopted providing for preliminary coagulation of the proteins by heat in a faintly acid solution and evaporation of the filtrates to a small volume. The trace of proteins escaping the first precipitation is then removed by a precipitant which does not appreciably increase the amount of salts in the final solution. The heat coagulation was carried out as suggested by Benedict.[9] The following procedure is recommended.

Into a flask introduce approximately 0.3 gm. of ground soy bean (20 mesh), add 3 to 5 cc. of water and 1 cc. of a 3 per cent solution of NaH_2PO_4, and let stand for a few minutes with occasional shaking. Run in a measured amount of blood (from 30 to 50 cc.) and let stand at room temperature for 30 minutes.

Heat 0.01 N acetic acid to boiling in a casserole, using five volumes of acid for one volume of blood. Run the blood from the flask slowly into the boiling acid and with constant stirring boil for $\frac{1}{2}$ minute. Add the same amount of boiling water, using this also to rinse the flask. Boil with stirring for 1 minute. Filter through a folded filter and wash the casserole three times with small portions of water (30 cc.), heating the water in the casserole in which the original coagulation took place and using a rubber-tipped stirring rod. The filtrate is boiled down rapidly over a free flame to about 10 cc. in a casserole. The contents of the casserole are now quantitatively transferred to a small graduated flask or cylinder, choosing the size so as to obtain nearly the volume of the original blood. Wash the casserole with the smallest possible amount of hot water three times. The volume in the flask or cylinder, after the final wash water has been added, should not be more than about three-fourths of the final volume.

At this stage of procedure different protein precipitants were tried. The first was a solution of colloidal iron (5 per cent Merck).

[9] Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 629.

This method, while removing the proteins completely, requires a little experience to obtain filtrates which can be evaporated to a very small volume without getting cloudy. It was therefore abandoned for the present. The next precipitant tried was 7 per cent phosphotungstic acid (Merck) in 2 per cent HCl solution, the phosphotungstic acid in turn being removed by gradual addition of small amounts of solid $Ba(OH)_2$.

This method gives very coarse, easily filterable precipitates, but the results are too low. As the phosphotungstic acid precipitant may possibly be used in some later work, some of the results obtained are given in Table VI. The results are compared with another procedure which will be discussed shortly.

TABLE VI.

Amino-Acid Nitrogen per 100 Cc. of Blood (Sheep).

Heat coagulation followed by phosphotungstic acid precipitation.		Heat coagulation followed by trichloroacetic acid precipitation.
mg.		*mg.*
4.64		
4.13		7.60
4.37		
4.48		
3.25		6.34
3.12		
Blood	3.18	
" + amino-acid	8.60	
Difference	5.42	
Calculated	9.32	

The third precipitant tried was trichloroacetic acid followed by kaolin, as suggested by Greenwald. The filtrate from the heat coagulation after being evaporated to a small volume and transferred to a graduated flask or cylinder is treated with trichloroacetic acid. Introduce into the graduated flask enough solid trichloroacetic acid to make an approximately 3 per cent solution. For this purpose the acid is either weighed out on a small scale in a little glass scoop or watch-glass and washed into the cylinder with a little water, or if several determinations are made, a 50 per cent solution of trichloroacetic acid is kept on hand and the corresponding amount of this solution is added with a Mohr

pipette. After making the solution up to volume, let it stand for 20 to 30 minutes. Shake with 2 gm. of kaolin, centrifuge, and run the supernatant liquid through a dry filter paper. A little kaolin always sticks to the side of the centrifuge tube above the liquid level and is carried along when the liquid is poured out. An aliquot part of the filtrate is transferred to a small flask, and a few beads and a drop of alizarin indicator are added. The liquid is brought to boiling over a micro burner and kept boiling very slowly (simmering) until the indicator turns.

The flask is removed from the flame and enough (1 to 2 cc.) of 1.0 N potassium hydroxide is added to make the liquid distinctly alkaline. Boil for 1 to 2 minutes, taking care that it does not boil over, because at this stage slight frothing and bumping occur. Make distinctly acid with acetic acid and boil down to the smallest possible volume. The liquid is now ready for the amino-acid apparatus. It is either transferred directly to the burette of the Van Slyke apparatus, washing the flask with very little water, or first transferred to a small accurately graduated test-tube and made to a definite volume. From this tube duplicates can be measured out by means of the burette of the amino-acid apparatus.[10] The latter procedure is especially recommended where large amounts of blood are available.

Table VII A shows a comparison between the direct trichloroacetic acid precipitation and the heat coagulation followed by the trichloroacetic acid precipitation. The corresponding results were obtained from the same blood each time. Table VII B shows the recovery of amino-acids added to the blood.

[10] The heat-trichloroacetic acid method gives filtrates which rarely exhibit any tendency to froth, when shaken in the deaminizing bulb. Should frothing occur, for some reason, caprylic alcohol, as recommended by Van Slyke, is very efficient. The best caprylic alcohol which we have been able to obtain at present gives such high corrections for the blanks that it should not be used without purification. For that purpose the alcohol is shaken twice (best in a separatory funnel) with a mixture of glacial acetic acid and $NaNO_2$ solution (30 gm. in 100 cc. of H_2O), the acid and the nitrate being in the proportion 1 : 5. The alcohol is then washed with a little water two or three times, transferred to a distilling flask, a very small fraction of NaOH added, and distilled under reduced pressure. The caprylic alcohol so purified shows a negligible increase in the blank figures.

TABLE VII.

A. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Trichloroacetic acid precipitation.	Heat coagulation followed by trichloroacetic acid precipitation.
	mg.	*mg.*
Sheep blood (oxalated)	7.82	7.43
Sheep blood (oxalated)	7.79	7.60
Sheep blood (oxalated)	7.50	6.34
Calf blood (oxalated)	7.60	7.24
Pig blood (oxalated).	8.33	8.37

B. Recovery of Added Amino-Acids.

	Blood.	Blood + amino-acid.	Difference.	Calculated.	Blood.	Blood + amino-acid.	Difference.	Calculated.
Sheep blood (oxalated)	7.50	16.33	8.83	9.32	6.34	15.84	9.50	9.32
Calf blood (oxalated)	7.60	16.85	9.25	9.40	7.24	16.44	9.24	9.40

The use of heat coagulation prior to amino-acid determination might seem objectionable on account of possible hydrolysis of protein during the process. Greenwald has shown in his publication that no splitting off of nitrogen takes place with his procedure, but here the first precipitation takes place in the cold. A glance at Table VII A shows that the heat coagulation-trichloroacetic acid procedure gives even slightly lower results than the direct Greenwald procedure. Recently Folin and Denis[11] have stated that, "All reagents involving heating are useless, because by heat (half an hour in a water bath) the nitrogen of normal blood filtrates may be increased to twice the real value." No figures are offered in substantiation of this statement. According to the statement of Folin and Denis, we should expect that the heat coagulation procedure would show much higher results in amino-acid nitrogen because the supposed increase in nitrogen would to a large extent be derived from protein hydrolysis.

[11] Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 492.

The above mentioned comparison seemed convincing, but an additional experiment was made to furnish further proof. Blood was precipitated with methyl alcohol according to Folin and Denis. Another sample of the same blood was precipitated according to Greenwald, and a third part of the blood was coagulated by heat and after evaporation treated with trichloroacetic acid and kaolin exactly as described above. On the filtrates, obtained by these three procedures, Kjeldahl determinations were made, using such volumes of filtrates as to represent about 40 cc. of blood, and the determination was repeated with three different samples of blood. Table VIII shows the results obtained.

TABLE VIII.

Non-Protein Nitrogen per 100 Cc. of Blood (Pig).

Methyl alcohol precipitation.	Trichloroacetic acid precipitation.	Heat coagulation followed by trichloroacetic acid precipitation.
mg.	*mg.*	*mg.*
22.93	23.88	23.60
29.92	32.03	30.80
28.15	30.81	29.22

SUMMARY.

The use of alcohol as a blood precipitant in the determination of amino-acid nitrogen in blood is undesirable.

A comparative study is made of several procedures to obtain suitable blood filtrates and their subsequent preparation for final analysis by the Van Slyke method.

The Greenwald method of blood precipitation has been found serviceable for amino-acid nitrogen determination. A modification of this procedure is also described.

Coagulation of blood at a boiling temperature in a weakly acid solution does not increase the filtrate nitrogen, provided the traces of protein which escape coagulation are properly removed.

A study of bloods of different species of animals and of human blood, both normal and pathological, is being made and will be published shortly.

In conclusion I wish to thank Mr. Isaac Neuwirth for his assistance in part of the experimental work.

STUDIES IN THE COMPARATIVE BIOCHEMISTRY OF PURINE METABOLISM.

III. THE PRESENCE OF ALLANTOIN IN MAMMALIAN BLOOD.

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(Received for publication, November 3, 1916.)

Since the urine of the great majority of mammalian species contains, as the end-product of purine metabolism, considerable quantities of allantoin, the presence of that substance in the blood also might reasonably be taken for granted. It seemed none the less desirable to convert this inference into certainty by the actual isolation of the substance. The attempt to effect this met with greater difficulty than I anticipated, but was finally so far successful that from the blood of the ox and the pig allantoin was obtained in quantity sufficient for positive identification, while its presence in that of the horse and the sheep was rendered practically certain. Human blood, on the other hand, yielded no evidence of containing even a trace of allantoin.

The method employed for the final isolation of the substance was that of Wiechowski.[1] But before it could be applied to blood it was necessary first to remove the proteins, and then to adopt some means of concentrating the other nitrogenous constituents (or at least the allantoin itself) into a solution of convenient bulk. In preliminary experiments with pig blood I sought to attain these objects by heat coagulation of the proteins, and evaporation of the faintly acid filtrate upon the water bath. The end-product obtained by the application of Wiechowski's method to this concentrate undoubtedly contained allantoin; but the amount was so small, and it was so contaminated by other materials, that its certain identification was hardly possible. Better results followed the adoption

[1] Wiechowski in Neubauer-Huppert, Analyse des Harns, Wiesbaden, 11th edition, 1913, ii, 1076.

of acid mercuric chloride solution[2] as a protein precipitant. This reagent had the advantage not only of giving easily and promptly a water-clear, protein-free filtrate, but also of introducing a metal (mercury) which could then be utilized to bring about the removal of the allantoin. To effect this it was sufficient to render the filtrate alkaline. The resulting precipitate contained, of course with many other substances, the whole or the greater part of the allantoin, and yielded, upon removal of the mercury, a solution of moderate volume, to which the method of Wiechowski could then be directly applied.

The alkalinity necessary for this first precipitation might be produced, it was found, by either sodium hydroxide or sodium carbonate. The former had the disadvantages of too readily precipitating mercuric oxide and of leading to the liberation, through reduction, of a considerable amount of metallic mercury. With the latter it was possible by avoiding a great excess to escape these inconveniences. Sodium carbonate therefore was the alkali finally adopted.

It is necessary here to correct an erroneous statement, current in standard works of reference,[3] to the effect that allantoin is *not* precipitated by mercuric chloride and sodium carbonate. The original source of this statement I have been unable to discover, but its inaccuracy is demonstrated by the following experiments.

From a solution of allantoin, of approximately 0.1 per cent concentration, there were measured ten 50 cc. portions. These were treated in pairs as follows:

1. Directly Kjeldahled.
2. Precipitated by addition of an equal volume of Wiechowski's reagent.
3. Precipitated by addition of 50 cc. of 0.5 per cent mercuric chloride followed by 2 cc. of saturated sodium carbonate.
4. As in No. 3, except that before precipitation there was added to the allantoin solution 2 gm. of sodium chloride.
5. Precipitated by addition of 50 cc. of 2 per cent mercuric chloride and 5 cc. of saturated sodium carbonate.

In Experiments 3 and 4 the precipitate was colorless; in No. 5 it was reddish brown, the white allantoin compound produced by the first portions of sodium carbonate being obscured by the basic oxide brought down by the later ones.

All the precipitates were collected after a 3 hour interval, washed with water, and Kjeldahled. The results of the nitro-

[2] Schenck, F., *Arch. ges. Physiol.*, 1894, lv, 203. Costantino, A., *Biochem. Z.*, 1913, lv, 419. Rosenberg, A. H., *ibid.*, 1914, lxii, 157. Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.

[3] Abderhalden's Biochem. Handlexikon, Berlin, 1911, iv, 1156.

gen determinations, expressed in cc. of 0.1 N ammonia, are given below.

1.	12.87, 13.00;	average	12.94
2.	12.61, 12.65;	"	12.63
3.	12.74, 12.76;	"	12.75
4.	12.73, 12.77;	"	12.75
5.	12.82, 12.78;	"	12.80

These data demonstrate sufficiently that, in spite of the statement to the contrary, allantoin is precipitated by mercuric chloride and sodium carbonate at least as completely as by Wiechowski's reagent. It appears further that the precipitation is not unfavorably influenced by considerable variations in the concentration of the reagents, or by the presence of even 2 per cent of sodium chloride. The last point has its importance in the present connection, for much sodium chloride will be produced in the neutralization of the acid filtrate from the blood-protein-mercury compounds.

The procedure which proved most satisfactory for the isolation of allantoin from blood may now be described in greater detail.

800 cc. of fresh oxalated blood were poured into 4,000 cc. (five volumes) of 2 per cent mercuric chloride in 0.8 per cent hydrochloric acid. The next day the mixture was filtered under suction, and 3,600 cc. of the colorless filtrate (corresponding to about 600 cc. of blood) were brought to a neutral or slightly acid reaction by the careful addition of about 90 cc. of 40 per cent sodium hydroxide. This neutralized filtrate was then treated with 20 per cent sodium carbonate in the proportion of 50 cc. to the liter. This produced a more or less dense white cloud, which in the course of 24 hours, if not earlier, settled down as a light yellow flocculent precipitate. The precipitate was separated as completely as possible by decantation and centrifugalization, suspended in water, and decomposed by hydrogen sulfide. The acid filtrate from the mercuric sulfide was evaporated to a bulk of about 25 cc.,[4] and treated exhaustively with 50 per cent phosphotungstic acid. The precipitate was removed by filtration through a bed of kieselguhr upon a Buchner funnel, and was thoroughly washed with 2.5 per cent phosphotungstic acid in 3 per cent sulfuric acid. The combined filtrate and washings were shaken with lead oxide till neutral, and basic lead acetate solution was added as long as further precipitation occurred. The insoluble lead salts were filtered off under suction and washed out

[4] At this stage there separated from the human blood, and from that only, a considerable quantity of crystalline material, which was easily identified as uric acid.

with water. The filtrate was acidified with acetic acid, and freed from chlorides by silver acetate. Upon removal of lead, silver, and hydrogen sulfide in the usual way, the final filtrate was concentrated to about 60 cc. and, after careful neutralization with chloride-free sodium hydroxide, was treated with an equal volume of Wiechowski's reagent. The immediate result varied from a flocculent precipitate in the case of ox blood to a faint opalescence in that of the human fluid. In every case a precipitate had settled out by the following morning. When this precipitate was subjected to the usual technique for the isolation of allantoin, it yielded a product highly impure, which it was difficult or impossible to bring to crystallization. It was therefore taken up in a very little water, treated again with a few drops of basic lead acetate, filtered, freed from excess lead, and precipitated a second time with Wiechowski's reagent. The solution resulting from the decomposition of this second precipitate was evaporated in a tiny glass basin, the dry residue moistened with a little water, and set aside to crystallize. When crystals formed they were washed with cold 50 per cent alcohol, which removed some still adherent greasy impurities, dried at 100°, and weighed.

The results obtained can be reported in a very few words. The blood of the *ox* yielded 4.7 mg. of clean well shaped crystals of considerable size and typical form, which melted, simultaneously with a specimen of pure allantoin heated alongside, at 232°C. The *pig* blood gave 3.4 mg. of crystalline material; the crystals were smaller, and assumed the shape not of perfectly pure allantoin, but of that substance as obtained by direct crystallization from urine; they showed, nevertheless, a melting point identical with that of the pure material. From the blood of the *horse* there were obtained crystals similar to those of the pig, although visibly inferior in quantity; unfortunately the dish containing them was accidentally broken before they could be weighed or their melting point determined. The *sheep* blood yielded at first only a smear, and it was only after much manipulation that there appeared two microscopic clumps of crystals; they had all the appearance of impure allantoin, but the quantity was too small to be accurately weighed, or even to serve for a satisfactory melting point determination. *Human* blood[5] furnished a minute

[5] The blood examined was a mixed specimen obtained by venesection from two nephritics. It is perhaps worth pointing out, in connection with recent observations by H. G. Wells (*J. Biol. Chem.*, 1916, xxvi, 319), how readily it yielded uric acid. The failure to obtain allantoin from such blood is even stronger evidence of its absence than if the blood had come from normal individuals.

amount of material, which could not be brought by any treatment whatever to yield crystals even remotely resembling allantoin.

The total yield of allantoin from all the specimens utilized was so small that no analysis of the material could be carried out. Nevertheless the identification, in the cases of the ox and the pig, resting as it does upon method of isolation, crystalline form, and melting point, may be regarded as beyond question. With the horse and the sheep, in the absence of a melting point determination, it is not so certain; although to one familiar with the microscopic shapes assumed by allantoin, little doubt upon the point would exist. The complete failure to find allantoin in human blood was of course to be expected.

The maximum yield of 4.7 mg. from about 600 cc. of ox blood represents something like 0.8 mg. per 100 cc. This is somewhat higher than the uric acid, and lower than the creatinine content of the same blood. It is of course merely a minimal value. It would indeed be ridiculous, with such a method as has been described, where so many bulky precipitates have an opportunity of adsorbing the substance sought, to lay any stress on quantitative considerations. It may, for instance, be entirely an accident that allantoin was isolated more readily and in greater quantity from the blood of the ox and the pig, than from that of the horse and the sheep. This being admitted, it is nevertheless suggestive that if we arrange the species in descending order in relation to the allantoin obtained, we get a series—ox, pig, horse, sheep, man—which almost coincides with the order taken by the same animals in regard to their capacity to form allantoin from uric acid, as judged by the relative proportions of these substances in the urine. Thus, the uricolytic index of the cow is 93, and of the pig 98, while that of the horse is 88, and that of the sheep only 80;[6] man, of course, has practically no allantoin-forming capacity at all.

Further investigation along the line suggested by these considerations must await the elaboration, if that should prove possible, of a more convenient method for the determination of al-

[6] Hunter, A., and Givens, M. H., *J. Biol. Chem.*, 1914, xviii, 403.

lantoin in blood. The only one at present available is so tedious and time-consuming that a repetition, with perhaps larger quantities of blood and a wider range of species, would hardly repay the labor involved. The main object of the present investigation was attained when allantoin, in however small a quantity, was actually isolated from mammalian blood.

THE IODINE CONTENT OF FOOD MATERIALS.*

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(Received for publication, November 8, 1916.)

The relation of iodine to thyroid metabolism has received a large amount of study, but the supply of iodine in food materials had been given no systematic attention until taken up by Forbes.[1] What influence its total absence in the diet might have on the development of an animal is problematic, but there has not been a lack of opinion that such an absence or low supply in the foods from certain regions was related to the development of goiter. To this opinion the work of Forbes and Beegle gives no credence. Further, we have learned that the production of hairless and weak pigs in certain regions in the northwestern part of the United States is attributed by some to a low supply or absence of iodine in the ration of brood sows.

Before the work of Forbes and Beegle appeared, we had under way a quantitative survey of the iodine content of a number of foodstuffs, waters, and samples of common salt used by farm stock. The results secured are in agreement with their data and show a low supply of iodine in plant materials and common salts, obtained from widely separate regions. Our only excuse for publishing these results is that they are confirmatory of the work of others, and in addition summarize our experience with certain methods proposed for the quantitative determination of iodine in organic materials.

The first method which we attempted to use was that of Krauss,[2] in which the iodine is determined colorimetrically as PdI_2. This

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Forbes, E. B., and Beegle, F. M., *Ohio Agric. Exp. Station, Bull. 299*, 1916.

[2] Krauss, R. B., *J. Biol. Chem.*, 1915, xxii, 115.

method is claimed to detect and determine as low as 0.0001 mg. of iodine in a 2 gm. sample. Though the directions were followed with the greatest care, no iodine, when added as potassium iodide in quantities of 0.0001 to 0.001 mg., could be recovered in the controls, and the method was finally abandoned.[3]

We next went back to the method proposed by Hunter[4] in which the iodine, after fusion and solution, is oxidized to iodate by sodium hypochlorite, the excess of chlorine removed by boiling, the iodine liberated from the iodate by potassium iodide in excess, and titrated with sodium thiosulfate. The results with this method were variable, at times being good and at other times disappointing.

Finally we tried the Kendall[5] method which is as follows:

> The substance, finely ground, is evaporated to a syrup in a nickle crucible with 5 cc. of 30 per cent sodium hydroxide and 10 to 15 gm. of solid sodium hydroxide broken into small pieces. The mass is then fused in a muffle furnace and any unoxidized organic matter is removed by small additions of solid potassium nitrate. After cooling, the melt is dissolved in water and transferred to a 500 cc. Erlenmeyer flask. 1 cc. of 10 per cent sodium bisulfite and a few drops of methyl orange are added, and the whole is made just acid with 85 per cent phosphoric acid. A few drops of bromine are added, the flask is well shaken, and the bromine boiled off by 10 minutes of vigorous boiling. About 10 drops of a 5 per cent solution of sodium salicylate are now added, and the flask and its contents cooled in running water. 5 cc. of 10 per cent potassium iodide are added, and a little phosphoric acid, if necessary, to bring about the iodide-iodate reaction. The liberated iodine is titrated against standard sodium thiosulfate, using starch paste as indicator. We found arrowroot starch as recommended by Hunter[4] suitable for this purpose. The iodine found must be divided by 6 to give the "original" iodine.

After some experimentation excellent results were obtained. The methyl orange end-point in the acidification with phosphoric acid was elusive, however, and a trial with Congo red showed the latter to be superior. In this case the acid is added until the indicator goes to a distinct blue, not stopping at the first break in the red which is still on the alkaline side. With the

[3] Mr. F. M. Beegle of the Ohio Experiment Station informed me that he also had been unable to obtain good results with the Krauss method.

[4] Hunter, A., *J. Biol. Chem.*, 1909–10, vii, 321.

[5] Kendall, E. C., *J. Biol. Chem.*, 1914, xix, 251.

above method, controls in which 0.1 to 1 mg. of iodine were added, the iodine was always recovered quantitatively. In the actual determination 2 gm. samples of the air-dried feedingstuff were used, and two and sometimes more determinations were made in nearly all cases. The minimum iodine content which Kendall claims can be detected is about 0.005 mg. of "original" iodine in a 2 gm. sample, or even 0.003 mg. of iodine. This much iodine, multiplied by 6 in the determination, gives a perceptible blue color with starch. In titrating these extremely small amounts the thiosulfate, which was standardized daily against potassium acid iodate, was used in various strengths of 0.002 N to 0.0005 N. Nothing is gained by using a strength weaker than 0.001 N, however, as the end-point becomes quite indistinct.

SUMMARY OF RESULTS.

Corn meal, tankage, commercial meat scraps, clover hay, alfalfa, cabbage, ground oats, oat meal, oats at the period of flowering, very young oats, June grass, timothy hay, wheat flour, sugar beet, milk powder, oil meal, distillers' grains, wheat gluten, oat straw, wheat straw, rape, cottonseed flour, peas, pea vine, and alfalfa grown in Kansas gave results which, on the whole, exclude the presence of as much as 0.003 mg. of iodine in 2 gm. of the substance. Wheat germ, barley, sweet clover, and Kansas-grown prairie hay possibly showed a trace of iodine, not more, certainly, than 0.005 mg. in a 2 gm. sample. Corn gluten, potato, lettuce, and the two natural waters examined showed a distinct trace of iodine, ranging from 0.003 to 0.01 mg. per 2 gm. of sample or 1 liter of water, respectively. The waters examined were from a local driven well and from Lake Mendota, a small inland lake which supplies this laboratory. 1 liter of water was evaporated to dryness with a little sodium hydroxide and the residue treated in the same manner as were the feeds. The blue color kept coming back at the final titration, however, and the iodine may have been liberated from the added potassium iodide by something other than iodate, as a ferric salt. This determination was originally intended for use with thyroids, which contain little inorganic material, and it is possible that some of the inorganic salts, which are present in rather large

amounts in these waters, rather than original iodine, liberated iodine from potassium iodide.

It was reported that some of the soils and waters in Kansas contain traces of iodine and it was considered of interest to determine the iodine content of some Kansas-grown feeds. Through the courtesy of Professor P. J. Newman of the Kansas State Agricultural College, samples of alfalfa, wheat, oat straw, and prairie hay were obtained and examined with results as noted above.

Samples of rock salt such as are commonly fed live stock were obtained and examined for iodine as follows: 10 gm. samples were dissolved in water and 10 cc. of saturated sodium carbonate solution were added, and 1 cc. of 10 per cent sodium bisulfite. A drop of Congo red brought the samples to the point in the Kendall determination where the phosphoric acid addition is made, and the determinations were finished as above. Controls showed this modification to be suitable for the determination of iodine in common salt. Samples were examined from two mines in Michigan, from New York, Louisiana, Kansas, Ohio, the Pacific Coast, and Salt Lake City. In no case was there any indication of the slightest trace of iodine present.

Attempts were also made to extract the iodine from 25 gm. of corn meal with a mixture of alcohol and acetone in a continuous fat-extraction apparatus, and determine the iodine in the evaporated residue. No iodine was found, however, and in a control only about three-fifths of the added potassium iodide was recovered.

An attempt was made to determine the iodine in 50 gm. of corn meal by adding the sample gradually to a large melt of sodium hydroxide, but the early production of sodium carbonate and subsequent solidification of the residue prevented the determination from being at all satisfactory, and a blank was obtained. A hot water extraction of 100 gm. of corn meal also gave a blank determination.

Distillations of the iodine from large samples suspended in 25 to 50 per cent sulfuric acid solutions were tried, but owing to the excessive frothing they have not yet been satisfactory.

TABLE OF RESULTS.

Substance.	No. of determinations.				Probable presence or absence of iodine.
	Total.	No iodine.	Trace.	0.003 to 0.01 mg. of iodine.	
Meat scraps	3	2	1		No iodine.
Tankage	2	1	1		" "
Corn meal	2	1	1		" "
Corn stover	2	2			" "
Cabbage	2	1	1		" "
Clover hay	2	2			" "
Very young oats	1	1			" "
Oats in flowering	1	1			" "
Ground oats	2	2			" "
Oat meal	2	2			" "
June grass	2	2			" "
Alfalfa	2	2			" "
Timothy	2	2			" "
Wheat flour	1	1			" "
Sugar beet	2	2			" "
Milk powder	2	2			" "
Wheat germ	1		1		Possibly a trace.
Distillers' grains	1	1			No iodine.
Wheat gluten	1	1			" "
Corn gluten	6	1	1	4	Undoubtedly a trace.
Barley	4	2	1	1	Possibly a trace.
Barley straw	3	2		1	No iodine.
Potato	3		1	2	Probably a trace.
Oat straw	1	1			No iodine.
Wheat straw	1	1			" "
Oil meal	1	1			" "
Lettuce	3		2	1	Probably a trace.
Rape	1	1			No iodine.
Cottonseed flour	1	1			" "
Peas	2	2			" "
Pea vine	2	2			" "
Sweet clover	1		1		Possibly a trace.
Water samples.					
Well water	2	1		1	Probably " " (?)
Lake Mendota water	3	1		2	" " " (?)
Kansas samples.					
Prairie hay	1		1		Possibly " "
Wheat	1			1	Probably " "
Oat straw	1			1	" " "
Alfalfa	1	1			No iodine.

TABLE OF RESULTS—*Concluded.*

Substance.	No. of determinations.				Probable presence or absence of iodine.
	Total.	No iodine.	Trace.	0.003 to 0.01 mg. of iodine.	
Salt samples.					
Michigan, No. 1	2	2			No iodine.
" " 2	2	2			" "
Louisiana	2	2			" "
New York	2	2			" "
Kansas	2	2			" "
Ohio	2	2			" "
California	2	2			" "
Utah	2	2			" "

DISCUSSION.

The above results are not absolute, as the table shows, but they are the best interpretation of the data obtained. That is to say, the results were not always in agreement—a blank and a trace of iodine being sometimes found in the same sample. The table gives the detailed results of the determinations. To test the effect of maturity, the oat plant was examined at three stages of growth, but negative results were obtained in every case.

It might be supposed that the objection to the method as applied to water would also apply to some of the feeds, but repeated controls showed that the method was quite applicable. Since 2 gm. samples were used, and 0.003 mg. of original iodine gives a blue color to the final solution, the samples which gave a blank had considerably less than one part of iodine in 650,000. In the case of the salts where 10 gm. samples were used, the amount of iodine was less than one part in 3,250,000. For the waters the amount was about one-hundredth of this. The substances, which showed a trace of iodine, had from about one part in 650,000 to one part in 200,000. Nearer than this it is impossible to say, the results, perhaps, being more qualitative than quantitative. There is, however, substantial agreement between these results and those secured by Forbes and Beegle.

SUMMARY.

1. Three methods for the determination of iodine in organic matter have been compared, with the result that the method proposed by Kendall was found to be by far the most accurate.

2. Iodine determinations have been made on many feeding materials, two natural waters, and several rock salts. In the majority of samples no iodine could be detected; some showed a trace of iodine to be present.

3. It would appear that the presence of iodine in feeding materials of vegetable origin is accidental and serves no necessary nutritive function in the plant. Further, the iodine requirements of animals must of necessity be met by the traces that occur in plant materials, waters, etc.

I wish to express my gratitude to Professor E. B. Hart of this Station, for the suggestion of this research and for much helpful advice and criticism.

COLORIMETRIC METHODS FOR DETERMINING SERUM CHOLESTEROL.

By PAUL G. WESTON.

(From the Laboratory of Pathology, State Hospital, Warren, Pa.)

(Received for publication, November 4, 1916.)

Colorimetric methods have been used by a number of workers for the quantitative determination of cholesterol in human blood. Weston (1) and Schmidt (2) used the Salkowski reaction. Grigaut (3), Autenrieth and Funk (4), Henes (5), Csonka (6), Bloor (7), Gettler and Baker (8), and others used the Liebermann-Burchard reaction. Each author used a different method of extraction. Autenrieth and Funk made fourteen determinations by their method and found an average of 1.278 mg. of cholesterol per 1 cc. of serum. Henes (5), testing some of the same serum by the Weston-Kent (9) method, found an average of 1.267 mg. per 1 cc. Weston (10) added known quantities of cholesterol to serum and recovered it quantitatively by the Weston-Kent method. Henes in the University of Freiburg used the Weston-Kent method for extraction and the Grigaut method for color determination; Windaus used his digitonin method, and Autenrieth used the Autenrieth-Funk method. The results were almost identical.

The experiment here described was undertaken in order to have a basis of comparison for the different methods both of extraction and of color determination. Starting with 100 cc. of human serum, I divided the quantity into two equal parts and added to one part 50 mg. of pure cholesterol dissolved in ether. The flask containing the serum-ether mixture was gently shaken from time to time until the ether had evaporated. By this method an even distribution of the cholesterol in the serum was obtained. A portion of each of these sera was extracted by each of six methods and each of the twelve resulting extracts

was tested by three methods. The following extraction methods were employed.

1. Bloor Method.—Three volumes of serum were added to seventy-five volumes of a mixture of alcohol and ether (alcohol, three parts; ether, one part) in a flask, and the mixture was heated to boiling. It was allowed to cool, after which sufficient alcohol-ether mixture was added to bring the volume to 100 and the extract was filtered. At this point the filtrate was divided into two parts. The first part was evaporated to dryness by means of gentle heat strictly according to Bloor's instructions, and the residue was taken up in chloroform for testing. The second was boiled with potassium hydrate for 2 hours and treated subsequently as in the Weston-Kent method.

2. Autenrieth-Funk Method.—Serum was digested on a water bath for 2 hours in ten volumes of 25 per cent potassium hydrate solution. The resulting liquid was extracted by adding to it twice its volume of chloroform and shaking vigorously for 5 minutes. The chloroform was then drawn off and a fresh lot added. This was repeated four times. The extract was dehydrated with anhydrous sodium sulfate and the clear solution used for testing.

3. Weston-Kent Method.—Serum was extracted with ten volumes of 95 per cent alcohol and kept at a temperature of 60°C. for 24 hours. The alcohol was filtered off and the residue washed twice with boiling alcohol. The residue was then further extracted for 24 hours with ether. The ether and alcohol extracts were combined and the mixture boiled with a few grams of potassium hydrate for 2 hours, at the end of which time the fluid had evaporated to a very small volume. A saturated solution of calcium hydrate was added and the precipitate which formed was collected on a filter, washed with calcium hydrate solution, dried, and extracted with chloroform.

4. Csonka Method.—2 cc. of serum, 20 cc. of alcohol, 4 gm. of potassium hydrate, and 2 cc. of a 10 per cent solution of barium chloride were placed in a flask of special construction and boiled under a reflux condenser for 1 hour. The mixture was extracted with ether and the extract washed with water until the wash water was no longer alkaline. The ether extract was then filtered into a flask, the ether was distilled off, and the residue taken up in chloroform for testing.

5. Gettler and Baker Method.—5 cc. of serum were allowed to run onto a loose coil of fat-free absorbent paper; this was dried over P_2O_5 at 55–60°C. *in vacuo* for 16 hours (over night). The coil was extracted in a Soxhlet with anhydrous ether for 5 hours. The ether was evaporated and the residue taken up in chloroform for testing.

6. Author's Method.—2 cc. of serum and 20 cc. of 25 per cent potassium hydrate solution were boiled on a hot plate for 2 hours. Water was added occasionally. At the end of 2 hours the solution was evaporated almost to dryness and a saturated solution of calcium hydrate added. The resulting precipitate was collected on a filter, dried, and extracted with chloroform.

Methods of Testing the Extracts.

1. Bloor Method.—A portion of each extract representing 0.3 cc. of serum was brought to 5 cc. by the addition of chloroform. 2 cc. of acetic anhydride and 0.1 cc. of sulfuric acid were added and the mixture was shaken. A standard tube was made by dissolving 0.0027 gm. of cholesterol in 10 cc. of chloroform. 4 cc. of acetic anhydride and 0.2 cc. of sulfuric acid were added and the mixture was shaken. The tubes were left in the dark at room temperature for 15 minutes. Readings were made in the Autenrieth-Königsberger colorimeter.

2. Autenrieth-Funk Method.—A second portion of the extracts, representing 0.1 cc. of serum, was treated in the same manner as above described except that immediately after shaking the mixture of chloroform, acetic anhydride, and sulfuric acid, the tubes were placed in a water bath at 35°C. for 15 minutes. The standard tube contained 0.0012 gm. of cholesterol. Comparisons were made in the Autenrieth-Königsberger colorimeter.

3. Weston Method.—A third portion of the extracts was placed in small tubes 100 x 10 mm. and of exactly the same diameter. Three dilutions of each extract were made representing 0.075, 0.1, and 0.125 cc. of serum. The contents of each tube were brought to 1 cc. by the addition of chloroform. In another set of similar tubes were placed quantities of cholesterol varying from 0.0001 to 0.0003 gm. in 1 cc. of chloroform, increasing by 0.000025 gm. 0.1 cc. of sulfuric acid was added to each tube and the tube was thoroughly shaken. All tubes were placed in the dark for 30 minutes, after which 1 cc. of chloroform was added to each tube. They were again placed in the dark for 15 minutes, after which comparisons were made and the amount of cholesterol determined.

A duplicate set of this extract was set up and treated in the same manner except that the standard was made up in 6 cc. of chloroform. Comparisons were made in the Autenrieth-Königsberger colorimeter. The findings by the colorimeter were the same as the results obtained by the use of the small tubes.

The results of all the tests are shown in Table I.

It may be seen that the color values obtained by Bloor's method are remarkably inconsistent and that the addition of cholesterol to the serum is in no case indicated by the readings. Whatever the method of extraction, there is nothing to show that the added cholesterol was recovered. The Autenrieth-Funk method shows clearly the recovery of the added cholesterol in every instance except in the extracts obtained by Bloor's method.

The values obtained by the author's colorimetric method were practically identical with those obtained with the Autenrieth-Funk method.

TABLE I.

Cholesterol per 1 Cc. of Serum.

Method.	Serum.	Colorimetric methods.		
		Bloor.	Autenrieth-Funk.	Weston.
		mg.	*mg.*	*mg.*
Bloor	A*	2.12	2.40	
	A**	1.89	1.62	1.73
"	B	1.71	2.88	
	B**	1.56	1.88	2.70
Autenrieth-Funk	A	2.12	1.86	1.84
	B	1.89	2.87	2.85
Weston-Kent	A	3.83	1.84	1.85
	B	3.11	2.86	2.85
Csonka	A	2.16	1.85	1.84
	B	2.22	2.87	2.83
Gettler-Baker	A	3.51	1.83	1.84
	B	3.00	2.85	2.85
Author	A	3.83	1.85	1.85
	B	3.96	2.87	2.86

* Serum A is plain serum; Serum B is the same with 1 mg. of cholesterol added to each cc.

** Extract saponified by Weston-Kent method.

It will be noted that Bloor's method of extraction did not give consistent results even when tested by other methods, and that the saponification of the extract did not affect the results appreciably. In the writer's hands Bloor's method was useless.

Inasmuch as the other five methods of extraction give identical results, the selection of a method is a matter of personal preference. I prefer the sixth on the ground that it is simple and economical.

Bloor and Autenrieth and Funk mention difficulties arising from the admixture of a yellowish or brownish tint to the green solution in the chloroform containing the serum extract. The latter authors by dehydrating the chloroform with anhydrous

sodium sulfate were nearly always able to remove the interfering color. Csonka overcame the difficulty by adding barium chloride to the mixture before saponification. Bloor states that, "If the evaporation of the untreated alcohol-ether extract was carried out carefully so as to avoid heating the residue after it had reached dryness, the chloroform extract was colorless and after treatment gave excellent readings." Mueller (11) used Bloor's method and was unable to obtain a green color free from brown even when the extract was evaporated before an electric fan. I have had a similar experience, and in eighty extractions was not once able to obtain a specimen wholly free from a brownish color.

SUMMARY.

A quantity of human serum was divided and cholesterol was added to one part in the proportion of 1 mg. to 1 cc. Portions of both the pure serum and the treated serum were extracted by different methods and the extracts were tested by different methods for cholesterol. The author failed to obtain valid results by Bloor's method. The extraction methods of Autenrieth and Funk, Weston and Kent, Csonka, Gettler and Baker, and Weston showed uniformly consistent results, the added cholesterol being recovered quantitatively in each case. The Autenrieth-Funk and the author's colorimetric methods gave equally satisfactory results. A new method of extraction is described.

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THE MODE OF ACTION OF UREASE AND OF ENZYMES IN GENERAL.

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(Received for publication, November 18, 1916.)

Van Slyke and Cullen, in a paper with the above title, deduced a general expression for the kinetics of enzyme action.[1] In a discussion of this question between Professor Pegram, of Columbia University, and the writer, it appeared that in the development of the equations an assumption was introduced which unfortunately limits their validity and applicability to definite conditions which are realized only in special cases.

Van Slyke and Cullen follow the time required for an enzyme molecule to complete the cycle of the reaction; in the example used, the sum of the times required to combine with a urea molecule, and to throw it off as ammonia and carbon dioxide. This length of time may be perfectly definite for a molecule of enzyme going through the cycle once, but with a large number of enzyme molecules it is necessary to consider the *average* time of an enzyme molecule in passing through the cycle.

If, in considering such a large number, the same fraction of the number of molecules is considered to be in each state of the reaction, that is, a part is going through the first stage of combination with the urea molecules, and a part through the second stage of decomposition of the enzyme-urea complex into enzyme, ammonia, and carbon dioxide, then the deductions of Van Slyke and Cullen hold. As the reaction proceeds, with definite initial quantities of enzyme and substrate, the absolute quantity of enzyme remains constant but the quantity of substrate decreases. Under these conditions it is evident, from a consideration of the kinetics of the reaction, as the quantity of substrate

[1] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 146.

decreases, with the same absolute amount of enzyme in the sphere of action, that a decreasing fraction of the number of enzyme molecules will be passing through the first stage of the reaction in a definite time interval, or that the average time of the first stage of the reaction for an enzyme molecule will be altered.

The second step of the reaction, decomposition of the complex, follows the monomolecular reaction velocity law in which the amount decomposed or reacting is proportional to the amount present. The average time each enzyme molecule remains in this combination is constant. The actual concentration of this intermediate compound is, however, changing. The total average time an enzyme molecule occupies in passing through a complete cycle of the reaction is made up, therefore, of the average time required to combine with a substrate molecule, which may be variable as the concentration of the substrate changes, plus the average time of decomposition of the complex, which is constant.

If a steady state exists in the reaction the deductions of Van Slyke and Cullen will conform to the facts. This steady state can occur only if a constant fraction of the total number of enzyme molecules is passing through the first stage of the reaction. This is true in the definite case of a large excess of substrate present in the reaction mixture, but as far as can be foretold, in no other simple case.

The fact that the calculated values, using Van Slyke and Cullen's equation, agree with the experimental results cannot be used as proof for the validity of the assumptions involved in the theoretical deductions. The constants of the equation are obtained from the experiments to which the equation is later applied.

THE MODE OF ACTION OF UREASE AND OF ENZYMES IN GENERAL.

By DONALD D. VAN SLYKE and GLENN E. CULLEN.

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(Received for publication, November 18, 1916.)

Our equation, contrary to the statement of Falk in the preceding paper, is not, as it appears to us, limited by the assumption that "a constant fraction of the total number of enzymes is passing through the first stage of the reaction." The equation expresses the following conditions: that the enzyme performs the two acts, of combination with and decomposition of the substrate, in sequence, not simultaneously, and that the period required for combination varies directly as the dilution of the substrate (urea). As corollaries it also expresses the following: first, since the two acts occur in sequence, the proportion of enzyme molecules engaged in each varies directly as the relative periods required for the respective reactions; second, since the average period of combination increases with substrate dilutions, the *proportion of enzyme molecules in the first stage* (*i.e.*, engaged in combining with or waiting to combine with substrate) *increases continuously with increasing substrate dilution.* The equation as it stands, therefore, not only covers the condition of a variation in the relative proportions of enzyme in the two states of activity, but specifically expresses the manner in which the proportions vary under the influence of changing substrate concentration.

It is a pleasure to acknowledge Dr. Falk's courtesy in discussing with us his criticism prior to its publication, so that his view and ours on the point where we failed to agree can be here presented together.

THE INFLUENCE OF THE COAGULATION BY RENNIN UPON THE GASTRIC DIGESTION OF MILK PROTEINS.

By J. T. LEARY and S. H. SHEIB.

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(Received for publication, November 9, 1916.)

Owing to the divergent results obtained by previous workers regarding the influence of the coagulation by rennin upon the subsequent gastric digestion of milk proteins, this study was undertaken with the view of ascertaining if possible wherein the great differences exist. According to Hawk,[1] coagulation of the casein by rennin greatly retarded subsequent action of pepsin. In his experiments no foreign substance was added to the digestive mixtures. Abderhalden and Kramm,[2] using a different method wherein sodium oxalate was added to prevent the coagulation, found that thereby the activity of the pepsin was lessened, but to a very slight degree. This result they explained by the adsorption of the pepsin by the paracasein with a resulting digestion from both within and without the coagulum.

PROCEDURE.

In the following experiments Lilly's scale pepsin was employed, in an acidity of 0.2 per cent hydrochloric acid. To prevent coagulation 2 per cent sodium oxalate was used and toluene was added as a preservative. The digestions were incubated at 38° for varying periods of time. Whole milk was used in all the experiments.

At the conclusion of each period of digestion, the already acid solution was saturated with sodium chloride in substance, heated to boiling, and filtered hot. The coagulum was washed with

[1] Hawk, P. B., *Am. J. Physiol.*, 1903–04, x, 37.

[2] Abderhalden, E., and Kramm, F., *Z. physiol. Chem.*, 1912, lxxvii, 462.

hot 10 per cent sodium chloride solution to free it from adhering non-coagulable matter and the filtrate made up to 200 cc.

When milk itself is so treated a water-clear filtrate is obtained which gives no precipitate with either nitric acid or Roberts' reagent. Milk and boiled pepsin similarly treated give the same results.

In the experiments with boiled milk the heating was conducted under a reflux with constant agitation, which procedure prevents the formation of a pellicle, particularly if the agitation is continued until the milk cools to room temperature. The nitrogen content of the coagulum and of the filtrate was determined by the Kjeldahl method. The Sörensen titration method was employed to determine the amino nitrogen content of the filtrate.

Series I.

1.	cc.	2.	cc.
Milk	10	Milk	10
H_2O	10	Sodium oxalate	10
Pepsin solution	5	Pepsin solution	5
1.2 per cent HCl	5	1.2 per cent HCl	5

Pepsin solution, 250 mg. in 100 cc.

1. Permitted to clot before adding acid.
2. Clotting prevented by addition of oxalate solution.

The periods of digestion were as follows:

Nitrogen.

	1. mg.	2. mg.
½ hr. digestion.		
Residue	23.24	42.98
Filtrate	34.58	14.56
Amino N	1.568	0.448
1 hr. digestion.		
Residue	21.7	43.4
Filtrate	35.84	14.56
Amino N	1.624	0.56
1½ hr. digestion.		
Residue	18.62	41.44
Filtrate	39.2	16.8
Amino N	2.016	0.56
2 hr. digestion.		
Residue	16.9	39.62
Filtrate	40.88	18.76
Amino N	2.086	0.588

The results obtained in this series clearly show the relatively poorer peptolytic action upon milk in which sodium oxalate has been used to prevent coagulation.

In Series II boiled and unboiled milk were digested in the same manner.

Series II.

Raw.				Boiled.			
1.		2.		3.		4.	
	cc.		*cc.*		*cc.*		*cc.*
Milk	10	Milk	10	Milk	10	Milk	10
H_2O	10	Oxalate	10	H_2O	10	Oxalate	10
Pepsin	5	Pepsin	5	Pepsin	5	Pepsin	5
1.2 per cent HCl	5	1.2 per cent HCl	5	1.2 per cent HCl	5	1.2 per cent HCl	5

Pepsin solution, 1 gm. in 100 cc.

1 and 3. Clot was permitted to form before adding acid.

2 and 4. Clotting prevented by oxalate.

The periods of digestion were as follows:

Nitrogen.

1 hr. digestion.

	1. *mg.*	2. *mg.*	3. *mg.*	4. *mg.*
Residue	15.12	43.24	14.14	41.72
Filtrate	44.80	17.92	47.04	19.04
Amino N	2.80	1.12	2.66	1.26

2 hr. digestion.

Residue	14.42	33.74	19.46	26.18
Filtrate	45.92	26.88	41.44	32.48
Amino N	3.36	1.40	3.36	2.24

3 hr. digestion.

Residue				
Filtrate	42.56	42.00	48.16	45.92
Amino N	2.80	2.24	3.08	2.80

4 hr. digestion.

Residue	15.40	25.20	15.00	28.64
Filtrate	49.28	37.24	51.52	45.92
Amino N	3.64	2.24	3.36	1.96

In this series the retarded digestion in the oxalate solutions is pronounced. In Experiment 3 this retardation is least evident. The digestion of boiled milk was slightly more rapid than that of raw milk.

A different procedure was followed in Series III. No foreign substance, *i.e.*, no sodium oxalate, was added to the digestive mixtures. The formation of a curd of paracasein was prevented by a change in the manner in which the various ingredients were added.

Series III.

1.	cc.	2.	cc.	3.	cc.
Milk	10	Milk	10	Milk	10
H_2O	10	H_2O	10	H_2O	10
Pepsin	5	1.2 per cent HCl	5	A mixture of equal parts of 1.2 per cent HCl and pepsin solution	10
1.2 per cent HCl	5	Pepsin	5		

Pepsin solution, 250 mg. in 100 cc.

1. Pepsin permitted to coagulate milk before addition of acid.

2. Acid added to precipitate casein before addition of pepsin.

1. Coagulation took place in about 2 minutes, forming a thick heavy curd.

2 and 3. No clot of paracasein was formed, the mixtures remaining practically homogeneous.

Nitrogen.

$\frac{1}{2}$ hr. digestion.

	1. mg.	2. mg.	3. mg.
Residue	44.80	25.20	25.90
Filtrate	13.44	35.84	33.60
Amino N	0.336	1.29	1.40

1 hr. digestion.

Residue	40.74	19.32	20.16
Filtrate	16.80	38.50	35.84
Amino N	0.64	1.90	1.90

$1\frac{1}{2}$ hr. digestion.

Residue	36.88	11.20	13.58
Filtrate	20.40	44.80	41.44
Amino N	0.70	2.40	2.40

2 hr. digestion.

Residue	35.98	10.36	11.20
Filtrate	22.68	48.44	46.90
Amino N	0.70	2.75	2.75

This series shows a uniformly better digestion in the cases in which no paracasein curd was formed. Whether the acid is added

first, followed by the pepsin, or whether both are added simultaneously apparently makes but little difference in the results obtained.

Series III having shown that the results obtained when acid and pepsin were added in the order named, or as a mixture, were practically identical, the procedure of adding a mixture was discontinued.

Series IV.

	1. cc.		2. cc.
Milk	10	Milk	10
H_2O	10	H_2O	10
Pepsin	5	1.2 per cent HCl	5
1.2 per cent HCl	5	Pepsin	5

Pepsin solution, 350 mg. in 100 cc.

1. Permitted clotting by pepsin before adding acid.

2. Added acid to precipitate casein before adding pepsin. No curd of paracasein was formed.

Nitrogen.

½ hr. digestion.

	1. mg.	2. mg.
Residue	40.80	26.46
Filtrate	17.92	31.36
Amino N	0.42	1.68

1 hr. digestion.

Residue	33.32	20.86
Filtrate	24.64	36.96
Amino N	1.40	2.24

1½ hr. digestion.

Residue	29.26	18.48
Filtrate	29.12	39.20
Amino N	1.68	2.80

2 hr. digestion.

Residue	26.18	15.54
Filtrate	31.92	42.28
Amino N	1.82	3.08

The relatively better digestion in the absence of a paracasein curd is shown here as in Series III.

SUMMARY AND CONCLUSIONS.

1. Abderhalden's and Kramm's results, showing that clotted milk was more easily digested than milk in which coagulation had been prevented by the use of oxalate, were confirmed, but it is believed that the peptic digestion in the latter case was inhibited by the presence of the foreign oxalate solution.

It would appear that the internal digestion of the paracasein curd by adsorbed pepsin as set forth by Abderhalden and Kramm is of rather minor importance.

2. Using a slightly different method from that employed by Hawk, his conclusion is verified; namely, that pepsin exerts its action much more readily upon the protein constituents of milk in cases where no thick elastic curd of paracasein is formed.

3. The formation of a curd of paracasein depends upon two separate factors; *i.e.*, the strength of the HCl in the juice and the quantity of rennin present.

4. The peptic digestion of boiled milk is slightly more rapid than that of raw milk.

THE PREPARATION OF GUANIDINE.*

By J. SMITH SHARPE.

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(Received for publication, October 14, 1916.)

Levene and Senior[1] give a method used by them for the preparation of large quantities of guanidine. Their process, which is not new, seems satisfactory but the purity of the product when tested is not over 95 per cent.

For the past year we have been using a large amount of guanidine in various forms, and being unable to obtain any of the salts which usually come from Germany, we tried some of the various methods of preparation given in Beilstein's *Handbuch*, and adopted the following which not only gives a good yield but also a product of great purity. The method seems to have been first suggested by Delitsch and Volhard,[2] but they give no details. Guanidine may be obtained by heating dry ammonium thiocyanate in a short-necked Jena flask for 20 hours at a temperature from 190–200°C. For this purpose an iron or copper oil bath is necessary, using fairly heavy oil so as to ensure a temperature within the range stated.

Method.

About 100 gm. of dry ammonium thiocyanate are placed in the flask and immersed in the oil, which should be above the level of the crystals. The heat is then raised gently until the required point is reached. When the 20 hours have expired, the mass is allowed to cool, extracted with hot water, and filtered. The filtrate, which contains the guanidine thiocyanate, is evaporated

* The expenses connected with this work were defrayed by a grant from the Medical Research Committee.

[1] Levene, P. A., and Senior, J. K., *J. Biol. Chem.*, 1916, xxv, 623.

[2] Delitsch, G., *J. prakt. Chem.*, 1874, ix, 2. Volhard, J., *ibid.*, 1874, ix, 15.

FIG. 1. Crystals of guanidine picrate. Microphotograph. × 50.

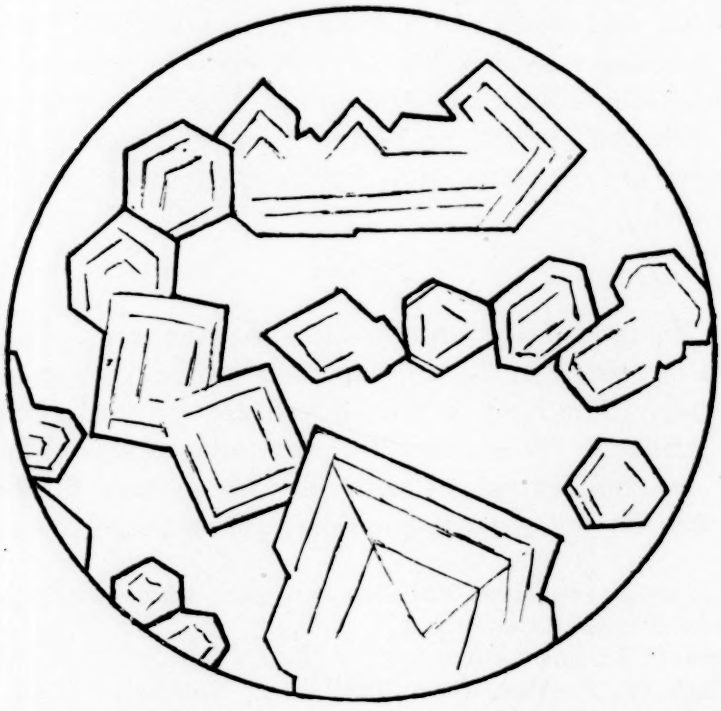

FIG. 2. Crystals of guanidine carbonate, produced by the process described. Camera lucida drawing. × 20.

to dryness and weighed, and for every one part of the thiocyanate present, 0.58 part of potassium carbonate is added, with sufficient water for solution. The whole is then evaporated once more to dryness. The potassium thiocyanate is then removed by repeated treatment with 90 per cent ethyl alcohol, decanting off after each addition, until a drop of the supernatant liquid fails to give a red color with perchloride of iron. Crude guanidine carbonate is obtained by this means, as it is insoluble in alcohol. It can be further purified by dissolving in a small quantity of water and adding 90 per cent alcohol to about five or six times the original volume, and allowing to settle, when pure guanidine carbonate crystallizes out.

Free guanidine may be obtained from this salt by treatment with the theoretical quantity of dilute sulfuric acid and barium hydroxide, but for convenience in use and preservation, the carbonate is the best salt.

Yield.—100 gm. of ammonium thiocyanate yielded 15 to 20 gm. of pure guanidine carbonate.

Tests of Purity.

1. Precipitation with sodium picrate: 0.1 gm. of prepared salt gave 0.158 gm. of picrate; theoretical 0.160 gm.

2. Nitrogen content of guanidine picrate formed.

	Calculated for $CH_5N_3.C_6H_3O_7N_3$:	Found: a.	Found: b.
N.....	29.11	29.50	28.77

3. Titration with 0.1 N sulfuric acid: 1 cc. 0.1 N H_2SO_4 = 0.009 gm. guanidine carbonate. 0.18 gm. taken (indicator, methyl orange) required 20.01 cc. of acid; *i.e.*, 20.01 × 0.009 = 0.18009 gm. guanidine carbonate.

PROTEINS OF THE CENTRAL NERVOUS SYSTEM.

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INTRODUCTION.

Although many workers have applied themselves to the elucidation of the chemistry of the nervous system, complete qualitative and quantitative studies of the proteins of the brain and nerves are lacking. This is due in great part to the impossibility of extracting from the fresh moist tissue more than a very small fraction of the total protein present. Even the examination of such extracted protein is attended with difficulties presented by the tenacity with which lipoid substances and inorganic salts are held by the proteins, making the problem of purification a serious one. The ease with which protein substances undergo change in the presence of solvents used for their extraction or in process of separation and purification further involves the problem. At the basis of a complete qualitative and quantitative analysis of the proteins lies the necessity of separating the proximate constituents of the tissue. This must be performed in such a way that the properties of the proteins may be unchanged. The present research has for its object the elaboration of a method that would accomplish the isolation of the individual proteins, and the application of such a method to the investigation of the

* At the time of his death Mr. McGregor was continuing certain phases of the investigation on the proteins of the brain. He had planned an elaborate study of his problem, involving not only a complete characterization of each protein but their amino-acid contents. In the development of this work he displayed unusual initiative; many of the points discovered were due to his independent study of them. In preparing his reports for publication his ideas and language have been scrupulously kept, though some of the matter reported is obviously preliminary.

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characteristics and relative amount of the proteins in the central nervous system.

The relations of the proteins to the functioning of the nerve cells is not understood. About 37 per cent of the dry substance of human brain tissue consists of proteins, and it is therefore to be anticipated that proteins play a relatively important part in the specific activities of the tissue. Scott[1] and Macallum,[2] in considering the nature of the Nissl substance of the nerve cells, regard it as axiomatic that an iron-holding nucleoprotein is necessary for the cell to carry on its normal function.

In considering the protein problem, the possibility was in mind that the different divisions of the human encephalon might be found to contain different proteins, and that the different species in the animal kingdom might exhibit characteristic differences in their brain proteins. Evidence of such modifications of substances has been gained by Reichert and Brown[3] in investigating the bloods of different animals.

The excellent method devised by Koch[4] for the estimation of the proximate constituents of animal tissues is the most efficient analytical scheme available for tissue work. It should be pointed out that this method is limited in its possible significance by our inadequate knowledge of the individual proteins. However, on the basis of the investigations of Halliburton,[5] Levene[6] and Ewald and Kühne,[7] on individual proteins Koch has divided the proteins into three fractions, simple proteins, nucleoprotein, and neurokeratin, the division in the case of the first two substances being accomplished by calculations based on the phosphorus content of the nucleoprotein. In the method of Koch the assumption is made that the proteins reported by Halliburton, together with the neurokeratin discovered by Ewald and Kühne, constitute all the proteins of the central nervous system. The

[1] Scott, F. H., *Tr. Canadian Institute*, 1899, vi, 405.

[2] Macallum, A. B., *Proc. Roy. Soc. Canada*, 1908, Series 3, ii, Section 4, 145.

[3] Reichert, E. T., and Brown, A. P., The crystallography of hemoglobins, *Carnegie Institution of Washington, Publication No. 116*, 1910.

[4] Koch, W., *J. Am. Chem. Soc.*, 1909, xxxi, 1329.

[5] Halliburton, W. D., *J. Physiol.*, 1894, xv, 90.

[6] Levene, P. A., *Arch. Neurol. Psychopath.*, 1899, ii, 1.

[7] Ewald, A., and Kühne, W., *Verhandl. naturh.-med. Vereins*, 1877, i, 457.

fact that by Halliburton's method less than 3 per cent of the total protein can be isolated, while neurokeratin represents about 15 per cent of the total proteins, makes it uncertain that the unextracted protein possesses the same characteristics as that actually separated. Yet our knowledge of the quantitative relations to the total proteins of so important a substance as nucleoprotein is based on two phosphorus determinations by Levene[6] on a nucleoprotein obtained from sheep brains by a modification of Halliburton's method. It was hoped that information gained from a complete protein analysis would furnish a basis for the extension of the method of Koch.

EXPERIMENTAL.

The "Direct Solution" Method.

In previous investigations on the proteins of the brain the value of the results is greatly minimized by the fact that the proteins are accounted for so incompletely. In the present investigation the first attempts to isolate the proteins were made by experimenting with the solvent action of various salt solutions on the moist sheep brain tissue. In so far as this tissue had been studied by chemical methods the results had shown great similarity to those from human brain tissue. On account of this fact, and also in view of the availability of absolutely fresh sheep brains, this tissue was adopted for preliminary study. M/10 solutions of the following substances were used as solvents: NH_4Cl, $(NH_4)_2SO_4$, $BaCl_2$, $CaCl_2$, $MgSO_4$, K_2SO_4, $KAl(SO_4)_2$, KCl, $NaC_2H_3O_2$, $NaHCO_3$, Na_2CO_3, $NaCl$, $NaOH$, $Na_2B_4O_7$, $NaKC_4H_4O_6$, Na_2SO_4, $ZnSO_4$, $Al_2(SO_4)_3$.

Following the method of Halliburton, 100 gm. of fresh tissue after mincing in a meat grinder were macerated for 24 hours at room temperature with 2 liters of the above solutions, thymol being added as an antiseptic. The turbid liquid was filtered through a Buchner funnel with the aid of filter paper pulp. Examination of the filtrates showed that in the case of the neutral or alkaline solvents, addition of weak acetic acid produced a precipitate, which is equivalent to Halliburton's "nucleoprotein." After filtering off this first precipitate, a second amount of protein was secured by boiling the solution. The markedly acidic solvents, like $ZnSO_4$ and $KAl(SO_4)_2$ yielded slight precipitates on neutralization, which were soluble in excess of dilute alkali or acid. Later experiments showed this material

to be an acid metaprotein produced by long contact of the tissue with the acid solutions. When solutions of weakly acid salts like $MgSO_4$ were used, the protein not precipitated by acetic acid was found to be present in smaller quantity than in the solutions of neutral salts, and if the filtrates from the nucleoprotein were allowed to stand for 24 hours in contact with weak acetic acid, the second protein fraction in large part disappeared.

The quantity of protein extracted was estimated by nitrogen determinations on measured volumes of the clear filtrate:

1. N determination on first filtrate gives total N.
2. N determination on Solution 1 after precipitation with acetic acid: 1–2 gives nucleoprotein.
3. N determination on filtrate from 2, after boiling: 2–3 gives acid-soluble protein.

The following table shows that there are no very marked differences in the solvent properties of the individual neutral salts, the amount of protein extracted being about 1 per cent of the weight of the original moist tissue. The alkaline solvents extracted a larger quantity of protein.

Nitrogen from 100 Gm. of Moist Tissue.

Solvent.	Total N.	Nucleoprotein N.	Acid-soluble N.
	gm.	*gm.*	*gm.*
$MgSO_4$	0.509	0.160	0.05
NaCl	0.480	0.131	0.047
K_2SO_4	0.558	0.165	0.043
KCl	0.544	0.181	0.044
$NaC_2H_3O_2$	0.450	0.101	0.056
$NaHCO_3$	0.698	0.191	0.071
NaOH	1.5	0.507	0.061
$ZnSO_4$	0.262	0.0	0.034
$KAl(SO_4)_2$	0.670	0.0	0.114

The above method of obtaining the proteins by direct extraction of the moist tissue was found to have many disadvantages that render it inapplicable to the quantitative study of nervous tissue proteins. These disadvantages may be briefly stated.

1. Small yield of protein.

2. Difficulty of filtration. This is one of the chief troubles encountered in the extraction of proteins, whether of plant or animal origin. Various means, such as that suggested by Os-

borne,[8] have been used to overcome it. The attempt was made to clarify the solution by means of aluminium cream, and by centrifuging, but so great a loss of protein occurred through occlusion or adsorption in the precipitate that these means were abandoned. Levene's[6] method of removing the suspended material by shaking with ether was not found to be very efficient.

3. Length of time required for extraction. 24 hours is required for extraction, and a similar period for precipitation and filtration. This gives opportunity for bacterial and hydrolytic changes.

4. The presence of lipoids in the precipitated protein. Halliburton reports lecithin as the most abundant impurity, while Levene states that "it is next to impossible even after continuous extraction with hot alcohol and ether during several weeks to get the product in such a condition that the evaporated alcohol or ether would leave absolutely no residue."

The Method of Drying and Extraction of Lipoids.

In view of the unsatisfactory results obtained by the above method of direct solution of the moist tissue, it was thought advisable to remove the lipoids as far as possible, in the hope that the proteins would thus be rendered more soluble, and the quantity of impurities minimized. This made it necessary to dry the tissue to prepare for the removal of lipoids by organic solvents. An objection that in the drying process similar changes might take place to those which vitiated the direct solution method was a serious one, and therefore to avoid all possibility of change in the tissue except that caused by loss of water, the following procedure was used.

Fresh sheep brains were ground in a meat chopper and spread in thin layers on large watch-glasses, then placed in a desiccator containing $CaCl_2$. The desiccator was surrounded by a freezing mixture. After the tissue was frozen the desiccator was continuously evacuated by a water pump for about 40 hours, by which time the tissue was found to be dry. It was ground in a mortar, then 20 gm. of the powder were treated with 300 cc. of benzene and the mixture was shaken in a glass-stoppered bottle on a mechanical shaking apparatus driven by a $\frac{1}{6}$ horse power motor. After shaking for 3 hours, the yellow benzene extract was filtered off and

[8] Osborne, T. B., and Campbell, G. F., *J. Am. Chem. Soc.*, 1900, xxii, 413.

a second and third extraction made. After three periods of agitation the residue was dried and weighed, the product being a nearly white powder which weighed 70 per cent of the weight of the original dry tissue. Further extraction with benzene yielded insignificant quantities of lipoids. Portions of the protein residue were shaken with water and with salt solutions, when a very great increase in solubility over the original moist tissue was noted, about 6 per cent of the original dry tissue being extracted as protein.

One of the difficulties inherent in the old method remained, however, in that the aqueous solutions contained much material in suspension and filtered very slowly. It was thought that a completer extraction of the lipoids would prevent this turbidity, and a systematic search was made for a solvent that would remove the maximum amount of lipoid by cold extraction. By conducting the benzene extraction at a temperature of about 40° the percentage of lipoid extracted was increased to about 40 per cent. Various mixtures of organic solvents, such as acetone, pyridine, or absolute alcohol, in benzene, were also found to be more efficient than pure benzene. The following table shows some of the solubilities of lipoids in the different solvents experimented with. Different samples of tissues were used in the various cases, but the general increase in solubility when alcohol is added to the benzene is shown clearly.

Solvent.	Dry tissue before extraction.	Residue after extraction.	Lipoid.
	gm.	*gm.*	*per cent*
Benzene	40.7	28.8	29.2
"	200	131.5	34.2
"	66.2	44.8	32.8
"	50	36.2	27.6
" at 40°	20	12.25	38.75
" " 40°	63.5	39.34	38.05
" 10 per cent acetone	20	13.85	30.9
" 10 " " aniline	20	14.45	27.75
" 5 " " alcohol	20	11.20	44
" 5 " " "	20	11.49	42.55
" 5 " " "	20	11.00	45
" 15 " " "	20	11.36	43.2
" 25 " " "	20	11.25	43.8

Thus a 5 per cent solution of absolute alcohol in benzene is found to extract in 10 hours about 45 per cent of the dry tissue.

That this solvent does not interfere with the solubility of the proteins is shown by the following experiment.

Three similar samples of air-dried sheep brain were extracted with different solvents, (1) benzene, (2) equal volumes of benzene and acetone, (3) 5 per cent absolute alcohol in benzene. The residues after complete extraction were shaken with solutions of 2 per cent NaCl, equal volumes of the filtrate were coagulated by heat, and the protein coagula weighed. The following table summarizes the result.

Lipoid solvent.	Dry tissue.	Tissue after lipoid extraction.	Lipoid extracted.	Protein extracted by NaCl. Dry tissue.
	gm.	*gm.*	*per cent*	*per cent*
Benzene	20	13.52	32.4	5.04
Benzene-acetone	20	13.25	33.7	5.25
Benzene-alcohol	20	10.23	48.8	5.04

The benzene-alcohol mixture, although extracting a much larger quantity of lipoid, does not cause a greater amount of protein to become soluble. It may be supposed that the 5 per cent protein obtained represents the amount of protein in the tissue that is soluble in neutral solvents. This point will be further considered when the quantitative relations of the proteins are discussed. A solution of 5 per cent absolute alcohol in benzene was adopted for removal of lipoids, and will be termed the "lipoid solvent."

The difficulty of filtering the aqueous extracts was noticed to increase when the temperature of the solvent water approached the temperature of the room. Recourse was made to cooled water for use in extraction, and with this expedient a nearly clear solution was obtained upon extracting the tissue, which filtered very readily through a Buchner funnel, using ordinary filter paper.

By the use of the freezing method above described it is possible to work with only small amounts of material. This method is free from objection as far as its effect on the tissue is concerned and may therefore be used as a check on the air-drying method here described for preparing large quantities of tissue.

To dry large quantities, hashed brain tissue after addition of 0.1 per cent thymol was spread in thin layers on large glass plates, which were

placed on shelves in a drying chamber through which air was drawn by an electric fan. By turning the tissue frequently so as to prevent film formation, drying can be accomplished in 20 hours, or in less time, depending upon the thickness of the layers of tissue. The material can then be powdered and desiccated over $CaCl_2$ *in vacuo* to remove the last traces of moisture. To compare the solubility of the proteins obtained by the freezing and air-drying methods, hashed sheep brain was divided into two portions, one of which was dried by the freezing method, the other in the air chamber. The dried tissue was then thoroughly extracted with the lipoid solvent. 3 gm. portions of the vacuum-dried and air-dried material were shaken with 300 cc. of 2 per cent NaCl, and two 50 cc. portions of each of the clear filtrates were taken for total protein determination.

	Protein in 50 cc.	
	Vacuum-dried.	Air-dried.
	gm.	*gm.*
Precipitated by excess alcohol	0.0820	0.0830
Coagulated by addition of acid at boiling point	0.0743	0.0737

This established the fact that air-drying does not affect the protein of the tissue to any appreciable extent.

Preparation of Tissue by Lipoid Extraction Method.

Through arrangement with Armour and Company, Chicago, sheep brains from animals killed on the day of shipment were packed in ice and expressed to this laboratory, 24 hours being consumed in transit. The tissue was in excellent condition, and was free from blood except that contained in the pia mater. Lesem and Gies[9] used sheep brain tissue received 24 hours after the death of the animals, and report that no appreciable changes in the tissue could be shown to have taken place in this short period.

To test further this question of possible change in the tissue, the brain of a dog removed from the skull 7 hours after the animal was killed was sectioned along the longitudinal fissure, and one-half dried immediately in the air dryer, the tissue drying in 3 hours. The remaining half of the brain was left for 3 days in a refrigerator, then dried in the usual way. Each half was extracted separately with benzene, and 2 gm. portions of

[9] Lesem, W. W., and Gies, W. J., *Am. J. Physiol.*, 1902–03, viii, 183.

the residue extracted with 300 cc. of 10 per cent $MgSO_4$. Nitrogen determinations on equal volumes of the filtrates before and after coagulation of the proteins were made, to compare the amount of protein in the two extracts. The results are as follows:

	Fresh tissue.	Stale tissue.
	gm.	*gm.*
Weight before drying	28	28
" after "	5.9	5.9
" " benzene extraction	4.1	4.1
Nitrogen in 25 cc. before boiling	0.0077	0.0079
" after boiling	0.0041	0.0043
Protein N in 25 cc	0.0036	0.0036

This shows that if the tissue is carefully preserved in a cold place very little change takes place in the solubility of the protein.

The brains as received from Chicago were sectioned into three portions, cerebrum, cerebellum, and the medulla with the pons and optic lobes. The tissue from the cerebrum was used in all the following experiments, except where otherwise stated. A very simple and effective means was used to remove the pia mater with blood vessels. A single cerebrum was placed in a mortar and gently tapped with a pestle until the membrane was loosened. The entire membrane could then be picked up with forceps and removed. Occasionally portions of the more closely adherent membranes remained, but these small amounts were removed by sieving the tissue after drying. The membrane- and blood-free tissue was passed through a meat grinder, mixed thoroughly with 0.1 per cent thymol, and spread in thin layers on glass plates which were placed in the drying chamber. After drying, the tissue was shaken with the lipoid solvent (1 liter to 100 gm.), filtered through cheese-cloth, dried from benzene in the air dryer, ground in a coffee mill, and passed through a 20-mesh sieve. Small amounts of membrane which had not been removed from the original tissue were retained on the sieve. The powdery product was next extracted for two 3 hour periods with the lipoid solvent, when a yellow, nearly odorless powder resulted on drying, which yielded only traces of lipoids when further treated with the solvent. As is usually found with dry protein powders, the product was extremely hydroscopic. If allowed to remain exposed to the air of the laboratory it rapidly took up water, gaining about 10 per cent in weight without at all losing its powdery appearance. Such moisture-containing protein slowly decomposed, as could be recognized by the development of slight odor and the change of solubility shown by the proteins. The protein powder thus prepared was kept in a vacuum desiccator over $CaCl_2$ and can thus be preserved for months without apparent change in its properties.

General Method of Studying the Proteins.

Various groups of proteins may be differentiated from others by differences in their solubilities. By the use of different solvents, it was proposed to divide the proteins into groups, then investigate the character of the protein or proteins in each group. The solvents used were water, aqueous solutions of neutral salts, acid solutions, alkaline solutions, and alcohol. On account of the finding by Ulpiani and Lelli[10] of a nuclein extracted by chloroform, extracts were also made with this solvent.

Proteins Extracted by Neutral Solvents.

Solutions of the protein powder were made in 2 per cent NaCl, 2 per cent Na_2SO_4, 10 per cent $MgSO_4$, and in distilled water. The solvent was first cooled to about 10°, then shaken with the powder for ½ hour on the mechanical shaker. The resulting solution was strained through cloth, and the nearly clear liquid could then be filtered through ordinary filter paper in a Buchner funnel in about ½ hour. The importance of letting the tissue stand for as short a time as possible in contact with the solvent was shown by extracting 12 gm. of the protein powder with 1,500 cc. of distilled water. Three 100 cc. portions of the filtrate were taken, the first portion coagulated by heat, and the protein weighed. To Portion 2 were added 10 gm. of $MgSO_4$, and the solution placed, together with Portion 3, in a refrigerator, and left 48 hours. The protein was then estimated by heat coagulation, and in each case less protein was found in the solutions which had been allowed to stand.

	Protein. gm.
Portion 1. Aqueous solution	0.0816
" 2. $MgSO_4$ "	0.0735
" 3. Aqueous "	0.0807

It is seen that the presence of the slightly acid salt $MgSO_4$ accelerates the decomposition of the protein.

The solutions of the proteins prepared as above described were studied by precipitation of the protein with weak acetic acid, total heat coagulation, fractional heat coagulation, fractional salting out, precipitation by alcohol, and by dialysis.

a. Precipitation by Acids.—It had been observed by Halliburton[5] that from solutions of brain proteins a precipitate is thrown down upon addition of weak acetic acid. In the case of salt

[10] Ulpiani, C., and Lelli, G., *Gazz. chim. ital.*, 1902, xxxii, 466.

extracts, he showed the presence of protein material in solution after the precipitation with acetic acid, but when distilled water was used as a solvent, no such protein was found. These results were confirmed in the present work in using the dried and lipoid-extracted tissue, with this important exception, that the distilled water solution held protein in solution after acetic acid precipitation. That is, the behavior of the distilled water solution was quite similar to that of the salt extract. The minimum concentration of acetic acid required to produce precipitation in solutions of lipoid-extracted tissue was 0.05 per cent for 2 per cent NaCl solutions, and 0.02 per cent for distilled water. The higher concentration which Halliburton found it necessary to use, 0.15 per cent, is perhaps attributable to the protective action of the lipoids present.

In comparing the ratio of protein precipitated by acetic acid to that left in solution when different solvents were used, it was found that the ratios depend on the concentration of acid used and on the medium in which precipitated.

This fact was observed by making a distilled water solution of the protein powder, 30 gm. to 1,000 cc. of water. The clear filtrate was divided into four equal parts. Part 1 was coagulated by heating to boiling in the presence of a trace of acid, and the total protein weighed. To Part 2, NaCl was added to make a 2 per cent solution; to Part 3, $(NH_4)_2SO_4$ was added to 10 per cent concentration, and Part 4 was left without salt addition. Each of Solutions 2, 3, and 4 was divided into two equal parts and different amounts of acetic acid were added. The precipitates were filtered off and weighed, and the protein in the filtrate coagulated and weighed. The following table shows the weights of the two fractions obtained, expressed in terms of gm. of protein from equal volumes of original solution.

Solution.	Concentrated $HC_2H_3O_2$.	Protein precipitated.	Protein in filtrate.	Total.
	gm.	*gm.*	*gm.*	*gm.*
1. Water				0.1045
2. 2 per cent NaCl	0.2	0.0615	0.0163	0.0778
	0.05	0.0544	0.0317	0.0861
3. 10 per cent $(NH_4)_2SO_4$	1	0.0912	0.00	0.0912
	0.05	0.0516	0.0431	0.0907
4. Water	0.02	0.0376	0.0620	0.0996
	0.1	0.00	0.0951	0.0951

It is apparent from the above table that no constant protein fraction is split off by acetic acid, and it is further significant that after treatment with acid the total protein is not recovered in the two fractions. The fact above noted as an exception to the finding of Halliburton, that distilled water holds a portion of the protein in solution after acid treatment, may here receive an explanation. In the older method, the acid used to precipitate the so called nucleoprotein was allowed to remain in the solution in contact with the soluble protein, and hydrolysis changed this protein over to metaprotein. This assumption has been tested on the lipoid-extracted tissue by allowing a solution from which a precipitate had been obtained by acetic acid to stand for a day, when it was found that a large proportion of the soluble protein had been changed to metaprotein and was precipitated on neutralization. It is evident therefore that the acid-soluble protein varies in amount according to the means taken to obtain it. The fact that it is soluble in distilled water is proof that it is not in any sense a true globulin.

b. Total Heat Coagulation.—The changes produced in the protein by small concentration of acid or change of medium are paralleled by changes produced by heat coagulation from different solvents.

$MgSO_4$, NaCl, and distilled water extracts were made and the protein coagulated by addition at the boiling point of sufficient acetic acid to make the concentration 0.01 per cent, this being the minimum concentration at which complete agglutination and precipitation would take place. Phosphorus determinations on the precipitated protein were made. Some of the values obtained from protein coagulated from different solvents are as follows:

Phosphorus.

H_2O	NaCl	$MgSO_4$
per cent	*per cent*	*per cent*
0.1557	0.0537	0.1538
0.1718	0.0756	
0.1395	0.0725	
0.1793	0.0663	
	0.0833	
	0.0560	

It is seen that the phosphorus content of the protein depends upon the method of isolating it from solution.

c. Fractional Heat Coagulation.—A 2 per cent Na_2SO_4 solution of the protein was made, to which were added 2 cc. of 1 per cent lactic acid per 100 cc., to agglutinate the protein when coagulation occurred. Five 25 cc. portions of the solution were placed in a water bath at an initial temperature of 45°. After 5 minutes Tube 1 was withdrawn and immediately placed in ice to cool. The temperature of the bath was raised to 50° for 5 minutes, at which point Tube 2 was removed. The heating was continued for 5 minute periods at the temperatures noted in the table below. After cooling, the precipitates were filtered off and weighed.

No. of 5 min. periods heated.	Temperature.	Weight of coagulum.
	°C.	gm.
1	45	0.0170
2	50	0.0280
3	55	0.0360
4	60	0.0440
5	70	0.0510

Another experiment using a solution of different concentration, where the heating intervals were 10 minutes, is noted.

No. of 10 min. periods heated.	Temperature.	Weight of coagulum.
	°C.	gm.
1	42.5	
2	45	0.0100
3	47.5	0.0215
4	50	0.0280
5	52.5	0.0300
6	55	0.0340
7	57.5	0.0350
8	60	0.0385
9	62.5	0.0400

Similar experiments have been repeated a number of times with substantially the same result, showing that a progressive coagulation takes place, no temperatures being noted at which excessive coagulation occurred which would point to the presence of a protein differing from the remainder of the solution. The phenomenon here observed is in accordance with the work of

Chick and Martin[11] who have shown that the heat coagulation of proteins is a reaction the velocity of which varies considerably with different proteins, and according to the acidity or salinity of the solution. It would appear from the data given above that the protein in solution is an individual and not a mixture. If the data of Halliburton are examined in the light of such studies on velocity of coagulation, the separation of three proteins by means of fractional heat coagulation may be given this same interpretation; *i.e.*, that the protein is a simple one and not three. The details of the method for fractional coagulation are not reported in Halliburton's paper describing the brain proteins; but in a previous study of serum albumin and serum globulin[12] a method is described which presumably was followed in the case of nervous tissue.

The solution to be coagulated was made slightly acid by the addition of 1 cc. of 2 per cent acetic acid to each 100 cc. "With regard to the length of time which a liquid has to be kept at any given temperature to ensure complete precipitation of the proteid at that temperature, it has been found that 5 minutes is as a rule sufficient." It is obvious from the above data and from the work of Chick and Martin that at any temperature a precipitate may be obtained, and if 5 minutes is given for a precipitate to form, the division into three proteins is easily explicable. The presence of considerable quantities of lipoids may also affect the speed and temperature of coagulation. This does not, however, furnish an explanation of the finding by Halliburton of only traces of phosphorus and nuclein in the fractions coagulated at 45° and 75°, while the 56° coagulating protein was rich in phosphorus and nuclein. Halliburton's data on the presence of nuclein are as follows:

Coagulation temperature.	Nuclein after gastric digestion.
°C.	*per cent*
47	2.13
56	5.26
75	Imponderable.

d. Fractional Salting Out.—Fractional salting out with $(NH_4)_2SO_4$ was done using solutions of proteins made with distilled water and with 2 per cent NaCl.

[11] Chick, H., and Martin, C. J., *J. Physiol.*, 1910, xl, 404.
[12] Halliburton, *J. Physiol.*, 1884, v, 152.

Five 200 cc. portions of the clear filtrates from each of these extracts were placed in 500 cc. measuring flasks, to which different amounts of a solution of 50 gm. of $(NH_4)_2SO_4$ in 100 cc. of solution were added. The solutions were then made up to 500 cc. and allowed to precipitate. 150 cc. portions were filtered off, and the protein was coagulated by heat; these coagula therefore represent the amounts of protein remaining in solution. The weights are given below under I. To 250 cc. portions filtered from each measuring flask 10 cc. of 1 per cent acetic acid were added. The weights of precipitates thus obtained are shown in Column II. 150 cc. of the filtrate from II were coagulated by heat; the weight of coagulum is recorded in Column III. Column IV shows the sum of II and III. The weights of the various fractions are calculated, for ease of comparison, on the basis of the total volume, 500 cc.

Solution.	$(NH_4)_2SO_4$ to 500 cc.	I. Total.	II. Acid-insoluble.	III. Acid-soluble.	IV.
	cc.	*gm.*	*gm.*	*gm.*	*gm.*
Distilled water.....	100	0.3362	0.1856	0.1042	0.2898
	150	0.2926	0.1476	0.1026	0.2502
	200	0.1866	0.0710	0.0878	0.1588
	300	0.1144	0.0280	0.0476	0.0756
	300	0.0536		0.0274	0.0274
2 per cent NaCl.....	100	0.5770	0.3082	0.1888	0.4970
	150	0.4960	0.2756	0.1832	0.4588
	200	0.3640	0.1862	0.1586	0.3448
	250	0.2280	0.0940	0.0978	0.1918
	300	0.1056	0.0320	0.0510	0.0830

It will be seen from Column I that this precipitation is of a similar additive nature to the fractional heat coagulation process. Columns II and III show increased amounts of protein precipitated with rising concentrations of $(NH_4)_2SO_4$, and confirm the conclusion from the data given on page 414, that on treatment with acid the total protein is not recovered in the two fractions. The ratios of precipitates shown by Columns II and III are not, indeed, constant, but the steady decrease of both sets of quantities with increased concentration of salt points to the probable presence of a unit substance before precipitation; the disparity between the ratios may be explained in the light of the results shown in the table on page 413, the ratio being in part dependent on the medium from which the precipitate is obtained. These results may be explained on the assumption that the protein extracted by water and by neutral salts is an individual and not a mixture, and that on treatment with acetic acid a cleavage

takes place, yielding a soluble and an insoluble protein product, while a third part of the original protein is not precipitated on boiling the solution.

Qualitative fractional salting out experiments with anhydrous Na_2SO_4 at 30°, where the relative volumes of the precipitates were noted, gave the same results as the above, the precipitates gradually increasing with rising concentration of Na_2SO_4. The results of two experiments in which NaCl extracts were salted out with $(NH_4)_2SO_4$ are given below. In Experiment I, cerebral tissue from beef brains, treated similarly to sheep tissue, was used. 40 cc. samples of the extracts were taken, to which were added different amounts of 50 per cent solution of $(NH_4)_2SO_4$, then made up to 100 cc. After precipitation, equal volumes of the filtrates were coagulated, and the weight of protein obtained.

Experiment I.		Experiment II.	
50 per cent $(NH_4)_2SO_4$ in 100 cc.	Weight of protein in filtrate.	50 per cent $(NH_4)_2SO_4$ in 100 cc.	Weight of protein in filtrate.
cc.	*gm.*	*cc.*	*gm.*
25	0.0399	30	0.0550
27.5	0.0377	35	0.0480
30	0.0356	40	0.0440
35	0.0307	45	0.0354
40	0.0240	50	0.0250
45	0.0182	55	0.0204

Two criticisms of the conclusions of Halliburton emerge from these data: first, that fractional salting out does not necessarily yield different products, as was assumed from the fact that $MgSO_4$ yielded precipitates at different concentrations. It is to be noted that no very definite line separated the proteins obtained by Halliburton by fractional salting out. The three fractions separated out at the following concentrations.

	Concentration of $MgSO_4$ at:	
	Commencement of precipitation.	Completion of precipitation.
	per cent	*per cent*
Fraction 1	30	50
" 2	50	90
" 3	80	100

The second objection lies in the effect observed when varying concentrations of acid are used, inconstant amounts of protein being precipitated. On this account it is impossible to assume that the protein precipitated represents a definite substance which existed preformed in the tissue and is wholly precipitated by weak acids. Levene[6] showed that the nucleoprotein obtained by him was extremely unstable: it rapidly lost its initial solubility in dilute alkalies and could therefore not be easily purified by this method. A small quantity which was dissolved in weak alkali and reprecipitated, immediately after the first precipitation with acetic acid, showed a phosphorus content of 0.45 per cent as compared with 0.56 per cent before such treatment.

e. Precipitation by Alcohol.—The differences exhibited by the proteins when obtained by different methods suggested the possibility of getting the protein intact by avoiding the use of heat or acid in separating it from solution. It was found that two volumes of alcohol added to one of the distilled water extract would cause ready separation of the protein. On determining the phosphorus content of such precipitates, a marked increase was found over the protein prepared in other ways, the phosphorus content varying from 0.7 to 1.2 per cent in different samples. Very little of this phosphorus could be removed by extraction with hot ether and alcohol, showing that the excess phosphorus was not due to phosphatides. By repeated extraction of the alcohol-precipitated protein with water, however, the phosphorus content was reduced to 0.11 per cent. Other analyses gave values between 0.11 and 0.14 per cent. The water contained phosphates directly precipitable by molybdate solution, but yielded a greater amount of phosphorus to molybdate solution after oxidation with nitric and sulfuric acids, showing that part of the phosphorus was in organic combination. A portion of the water extract was evaporated to dryness; the residue contained, in addition to inorganic salts, a small amount of organic material which was not again soluble in water, and which gave weak responses to protein color tests. The action of alcohol on the tissues apparently produces decomposition, and, further, results in the adsorption or occlusion of large amounts of phosphates. The protein after precipitation by alcohol could not be redissolved, so attempts were made to dialyze the phosphates and other impurities from the protein solution.

f. Dialysis.—500 cc. of the extract were dialyzed in a parchment vessel for 2 days against running water, a small quantity of thymol being added as a preservative. The protein was then precipitated by $(NH_4)_2SO_4$, when phosphorus determination showed a content of 0.718 per cent.

Part of this material was also tested for the presence of iron. Reference has been made earlier in this paper to the finding by histologists[1] that the Nissl bodies in nerve cells contain iron, apparently in protein combination. There is no record in the literature of iron being detected in the proteins after separation and purification. To test the protein for this element, small amounts were decomposed (1) by incineration in a porcelain crucible and (2) by oxidation with nitric acid. Control experiments were run, which gave negative results when tested with $K_4Fe(CN)_6$ and KCNS. The solutions obtained from the decomposed protein, however, yielded unmistakable precipitates of Prussian blue and red coloration with thiocyanate. The test for iron has been applied to the protein separated by coagulation and by salting out, and has always given a positive reaction.

Proteins Extracted by Acidic Solvents.

Acid extractions of the lipoid-extracted tissue were made with 1 per cent acetic acid and with 5 per cent solutions of oxalic, citric, and boric acids. The clear filtrate in each of these cases yielded a precipitate when neutralized. That the protein thus obtained represents a decomposition product of true tissue protein was made evident by completely extracting some of the dry tissue with 2 per cent NaCl solution, then shaking the residue with the solvents above noted. In no case did any precipitate appear on neutralizing these acid filtrates. Further, if to the 2 per cent NaCl solution 1 per cent of acetic acid be added and the solution shaken, a precipitate appears on neutralizing. Reference is made on page 414 to the slow formation of metaprotein by permitting the distilled water solution of the protein to stand in contact with weak acetic acid. This explains the appearance of the "new protein" obtained by Marie[13] by compressing fresh brain tissue to which had been added 1 per cent

[13] Marie, A., *Compt. rend. Soc. biol.*, 1911, lxx, 322, 459; 1911, lxxi, 709; 1912, lxxii, 100, 528.

of acetic acid, and for which he proposed the name "*antilyssine.*" It may be stated that such an acid metaprotein does not exist preformed in the brain.

Proteins Extracted by Alkaline Solvents.

Tissue which has been extracted as completely as possible with dilute salt solutions or distilled water invariably yields protein to a weak ammonia solution. This had been pointed out by Levene, in the direct extraction of moist tissue. Levene considered this protein as identical with the nucleoprotein extracted by dilute salt solutions. After thorough extraction with ammonia up to 1 per cent concentration, a further amount of protein is taken up by 0.5 per cent NaOH. The method of Schkarin[14] for the extraction of "neurostromin" is based on this fact. The protein extracted by NH_4OH or by NaOH is not precipitated upon neutralization, but rapidly deposits from solution when the acid concentration reaches 0.1 per cent acetic. No protein remains in solution after the precipitation by acid, in distinction from the nucleoprotein obtained from water extracts. Analysis of the protein after purification by resolution and reprecipitation shows a phosphorus content of about 0.6 per cent. A slight though definite reaction for iron is given by the purified substance. The protein is readily soluble in very dilute alkalies and does not lose this property even after continued standing in the presence of acetic acid. In this it differs from the nucleoprotein that is soluble in distilled water.

Extraction of the Tissue with Alcohol.

In order to settle the question of a protein similar to the prolamine group being present in nervous tissue, 10 gm. of the dried material were shaken with 300 cc. of 70 per cent alcohol. A slight residue on evaporation of the filtered alcohol gave negative reactions to all protein color tests applied.

[14] Schkarin, A. N., Inaugural Dissertation, St. Petersburg, 1902, i, 453.

Extraction of the Tissue with Chloroform.

Extraction of the dry tissue with chloroform was carried out in view of the finding of Ulpiani and Lelli,[10] confirmed by Steel and Gies[15] that the chloroform-water extract of fresh tissue contains nuclein. 30 gm. of dried tissue, before extraction with the lipoid solvent, was treated with 150 cc. of chloroform and shaken for ½ hour. To the yellow chloroform extract an equal volume of ethyl acetate was added, which produced immediately a pure white waxy precipitate. This precipitate was tested for protein in various ways, but all the tests, including Millon's and biuret which Steel and Gies had found positive, were negative.

Quantitative Relations of the Proteins.

It has been shown above that water and weak alkalies extract proteins from dried and lipoid-extracted nervous tissue, and from the means taken to obtain these substances it may be assumed that they exist preformed in the tissue and are not cleavage products produced by the action of the solvents. It has also been shown that acidic solvents do not extract unchanged proteins. The quantitative relations of the proteins remain to be considered.

Air-dried tissue from the cerebrum of the sheep brain, after thorough desiccation *in vacuo*, was quantitatively extracted with the lipoid solvent. The extracted material was ground in a mortar, then sieved through fine cloth until a nearly impalpable powder resulted. This was carefully dried *in vacuo* and a weighed sample taken for analysis. The ease with which the protein forms a sticky, unfilterable emulsion with solvents at room temperatures makes it imperative to work with solutions at about 10° when repeated extractions are to be made. In the following extractions, ice water was used in each case, and it was found that the solutions filtered readily after shaking for ½ hour. Three extractions of the weighed sample were made with distilled water, the residue being quantitatively recovered after each extraction. The protein in the extract was estimated by coagulating a measured volume in the presence of 0.02 per cent acetic acid, and weighing. By evaporating to dryness a measured volume of the extract, the weight of the total extracted material was obtained, and by difference the non-protein fraction calculated. 0.05 per cent NH_4OH was used to extract the residue from the third water extraction, and the

[15] Steel, M., and Gies, W. J., *Am. J. Physiol.*, 1907–08, xx, 378.

protein in this solution was weighed after precipitation by addition of 10 cc. of 1 per cent acetic acid to each 100 cc. of extract. The residue from NH_4OH was first treated with 0.05 per cent NaOH and the protein estimated in the same way as from the NH_4OH solution. The final residue was dried in the drying chamber, ground to a fine powder, and extracted with 0.1 per cent and then with 1.0 per cent NaOH, neither of which solvents removed any further protein. The following table shows the result of the analysis.

Solvent.	Original dry tissue.	
	Protein.	Non-protein.
	per cent	*per cent*
Benzene-alcohol		45.92
Water, 1st extraction	4.17	10.1
" 2nd "	0.539	0.1
" 3rd "	0.0	0.0
0.05 per cent NH_4OH	3.753	1.12
0.05 " " NaOH	5.906	6.32
	14.368	63.56
	63.56	
	77.928	

The residue from these extractions was treated with hot alcohol under a reflux condenser for 24 hours, but no residue was obtained on evaporation. It was then oxidized with HNO_3 and H_2SO_4 for phosphorus determination. 0.284 per cent phosphorus was found. The presence of phosphorus in the residue after complete extraction with lipoid and protein solvents is capable of several interpretations. First, it may be due to the presence of an insoluble phosphorus-containing protein. Second, it may be found that neurokeratin itself contains phosphorus, and, as suggested in the previous discussion of neurokeratin, the substance described by Ewald and Kühne may be a product resulting from digestive action on a phosphorus-containing protein which constitutes the true supporting tissue. Again, the protein may represent an unextracted protein which has become insoluble as a result of the processes to which the tissue has been subjected.

Proteins from Different Divisions of Sheep Brain.

It has been stated that the brain tissue used in most of the experiments described in this paper was prepared from the cerebrum of the sheep. Qualitative tests were made to determine

whether the different divisions of the brain of this animal contain similar proteins.

Four divisions of the brain were examined separately. The cerebrum was divided into two parts by section across the cruciate sulcus; also the cerebellum was separated from the medulla with pons and optic lobes. These four divisions were dried, extracted with the lipoid solvent, and the dry material was shaken with water and with salt solutions. Examination of the solutions showed the presence of a protein that yielded a precipitate with acetic acid and left a quantity of protein in solution which was coagulated on boiling. Thus the properties were similar to those exhibited by the protein from the whole cerebrum. By extraction with weak alkalies a second yield of protein was obtained, which possessed the same characteristics, as regards stability towards acids and absence of splitting products, as the alkali-soluble protein from the cerebrum.

Proteins from Nervous Tissue of Different Animals.

The brains of several animals other than the sheep have been examined qualitatively with respect to the proteins present, and will be considered separately.

Beef Brain.—Experiment I, page 418, shows the fractional precipitation by $(NH_4)_2SO_4$ of a saline extract of beef brain, where exact ly the same phenomena are observed as in the case of sheep tissue, and it may therefore be assumed that the protein of the beef brain soluble in neutral solvents is an individual substance. The distilled water extract of beef brain also gives a precipitate with dilute acetic acid, and yields more protein on boiling the filtrate. That protein soluble in dilute alkalies is present was shown by completely extracting the tissue with 2 per cent NaCl and subsequently shaking with dilute NH_4OH. The filtrate, on slightly acidifying with acetic acid, deposited a protein having the same general properties as the corresponding protein from the sheep brain.

Dog Brain.—Data are given on page 411 on the extraction of the proteins from the brain of the dog. This tissue was tested in a similar manner to beef brain. The distilled water extract yielded a soluble and an insoluble fraction on addition of acetic acid. Dilute alkalies extracted from the tissue a protein corresponding to that of the sheep brain.

Rabbit Brain.—Fresh brain obtained within a few minutes after the death of the rabbit has been analyzed for neutral salt-

soluble protein, and the result found positive. In the case of this tissue no subsequent extraction was made with dilute alkalies.

Human Brain.—A portion of the cerebrum from human brain tissue received about 48 hours after death was dried and the lipoids were extracted in the same way as from sheep brains.

20 gm. of the air-dried tissue extracted thoroughly with 5 per cent absolute alcohol in benzene left a residue of 11.49 gm., or 57.45 per cent of the dry tissue. The lipoid-extracted material was shaken with 2 per cent NaCl. Weak acetic acid was added to a portion of the filtrate, when protein material rapidly precipitated, and upon boiling the filtrate from this precipitate a further amount of protein was obtained.

This corresponds exactly with the properties of the protein from sheep brain. After complete extraction with NaCl solutions, the human brain yielded protein to dilute alkalies, precipitable upon acidifying.

DISCUSSION OF RESULTS.

The activity of the central nervous system presents such a complexity of reactions, involving the structure and intercommunications of ten billions of cells, and the constitution and properties of all the substances within the cells, that the relation of a single group of substances like the proteins to the integrated action of the tissue will be learned only through patient and persistent work on the problem.

The properties, however, of isolated constituents may furnish a clue to their functions in the tissues. It is from this standpoint, in part, that the results here presented are to be considered. It is found that a protein substance of extreme lability is present, the properties of which show it to be especially reactive to slight changes in acidity or alkalinity. The transmission of the nervous impulse along a fiber has been shown by Tashiro[16] to be accompanied by the production of CO_2, and we may therefore assume that the change in the nerve cell body which results from the entrance of the impulse might be initiated by a change in the H ion concentration in the cell substance. In the nucleoprotein described we may see, in the absence of more conclusive data,

[16] Tashiro, S., *Am. J. Physiol.*, 1913, xxxii, 107.

a substance possessing the instability requisite for quick response to such mild stimuli.

The fact that histological evidence points to the presence of two phosphorus-containing proteins in the nerve cells, may possibly be related to the isolation of two such proteins, one substance being soluble in neutral media, the other requiring very small concentration of alkali for its solution.

It is remarkable that the proteins obtained from the brains of various animals should be so similar in their properties as to give identical qualitative reactions. The same conclusion has been reached with regard to the inorganic constituents of brain tissue by Weil,[17] through a complete study of the ash of the human brain and that of the ox. He concludes that the psychical differences in the two species are due to different ratios of gray and white substance. In the case of the proteins, the absolute identity of the proteins in the different animals has not been shown; the significance of the quantitative method outlined in this paper lies in its application to the preparation of the various proteins, to permit of precise characterization.

SUMMARY.

I. A new method of preparing nervous tissue for quantitative study of the constituents, especially proteins, is described. The soluble proteins can be directly and completely extracted by suitable solvents, and quantitatively estimated.

II. The proteins of the central nervous system are at least three in number:

1. A phosphorus- and iron-containing protein soluble in distilled water. It comprises about 5 per cent of the dry brain tissue. The properties of this protein may be summarized in the following statements. (a) The protein extracted from brain tissue by neutral salts or by distilled water is precipitated in a progressive, additive manner by increasing concentrations of $(NH_4)_2SO_4$. (b) Fractional heat coagulation of such solutions shows a similar additive coagulation of the protein. (c) These data point to the individual nature of the protein. (d) The protein whether extracted by distilled water or by neutral salt

[17] Weil, A., *Z. physiol. Chem.*, 1914, lxxxix, 349.

solutions shows the same general properties. No true globulin can be said to be present. (e) The protein is very unstable. In the presence of low concentrations of acid it breaks down, yielding at least three products, the natures of which are dependent on the concentration of acid used and on the medium in which the products are precipitated. (f) The protein when obtained by total heat coagulation varies in character according to the solvent from which it is coagulated, and the amount of acid used to effect coagulation. (g) The protein obtained by different methods has always given a slight though definite positive reaction when tested for the presence of iron. (h) The phosphorus content is about 0.11 per cent.

2. A phosphorus- and iron-containing protein soluble in dilute alkalies. Approximately 10 per cent of the dry tissue is present in this form. (a) The protein extracted by alkaline solvents is qualitatively and quantitatively different from that extracted by neutral solvents. (b) The protein extracted by alkaline solvents may be completely precipitated by the addition of acid, and the protein is not decomposed in the precipitation. (c) This protein contains 0.60 per cent of phosphorus.

3. Supporting tissue, insoluble in neutral, acidic, or alkaline solvents. This makes up about 20 per cent of the original dry brain tissue.

III. The brains of the ox, rabbit, dog, sheep, and man contain, in general, similar proteins.

IV. The different divisions of the sheep brain contain similar proteins.

V. The central nervous system does not contain preformed the "globulins" described by Halliburton.[5]

VI. Acid metaprotein, described by Marie,[13] does not exist preformed in the brain.

A NOTE ON THE INFLUENCE OF INOSITE UPON THE EXCRETION OF PHENOL IN THE DOG.

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As a result of his work on the utilization of inosite, Anderson[1] found that in the dog, after feeding a known amount of inosite, he could recover in the feces up to 77 per cent of the amount, while the urine contained only minimal amounts. In man[2] 9 per cent was recovered in the urine while none at all could be demonstrated in the feces. The question as to what happens to the rest of the inosite is left open.

In accordance with the belief that inosite is a hexahydroxybenzene[3] and that inosite undergoes bacterial fermentation in the intestine it seems plausible to consider the possibility that the benzene fraction may be split off, thus giving rise to phenol.[4] Consequently it was thought worth while to try to demonstrate an increase in the phenol content of the urine after feeding inosite.[5]

The results of such feeding are shown in the table, and it is evident that no "extra" phenol appears in the urine upon the ingestion of inosite, so that the rôle which this substance plays in metabolism is still in doubt.

[1] Anderson, R. J., *J. Biol. Chem.*, 1916, xxv, 391.

[2] Anderson, R. J., and Bosworth, A. W., *J. Biol. Chem.*, 1916, xxv, 399.

[3] Hammarsten, O., and Hedin, S. G., Physiological Chemistry, New York, 7th edition, 1915, 579.

[4] Folin O., and Denis, W., *J. Biol. Chem.*, 1915, xxii, 309.

[5] The inosite was obtained through the kindness of Dr. Anderson.

Influence of Inosite on the Excretion of Phenol in the Dog.

Date.	Total N.	Phenols.				Inosite.			
		Free.	Total.	Free.	Conjugated.	Fed.	Recovered.		
							Urine.	Feces.	Percentage of amount fed.
1916		*gm.*	*gm.*	*per cent*	*per cent*	*gm.*	*gm.*	*gm.*	
Oct. 15	14.82	0.203	0.267	76.2	23.8				
" 16	14.89	0.200	0.270	74.2	25.8				
" 17	14.99	0.208	0.277	75.2	24.8	5.0	None.		
" 18	14.75	0.203	0.272	74.8	25.2			1.875*	37.5
" 19	14.81	0.202	0.267	75.8	24.2	20.0	0.114		
" 20	15.00	0.203	0.270	75.3	24.7			8.234†	41.2

* Feces of 17th and 18th.

† Feces of 19th and 20th.

As for the actual feeding and recovery of inosite, the results obtained corroborate those of Anderson.[1]

STUDIES OF INFANT FEEDING.

THE CHEMICAL CHANGES PRODUCED BY THE ADDITION OF LIME WATER TO MILK.

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(Received for publication, November 27, 1916.)

While the addition of lime water to milk used as infant's food has been common practice for many years, it has not been known just what changes the lime water produces, and as far as we know the subject has not been investigated from a purely chemical standpoint. The use of lime water in this connection has been based, first, upon the erroneous assumption that the acidity of cow's milk is much higher than that of human milk[1] and must be reduced before it can be used as a food for infants; and, second, upon the well known fact that lime water when added to milk in sufficient amount inhibits the curdling of the casein by rennin and hence, by deduction, will prevent the formation of casein curds in the stomach, a source of much trouble in infant feeding. As the addition of any alkali to milk causes a precipitation of insoluble calcium phosphate, a fact well known to those familiar with the chemistry of milk, and in view of the results recently obtained by us with reference to the availability of dicalcium phosphate when present as a constituent of infants' food, it seemed desirable that more definite information should be obtained concerning the chemical changes produced by the addition of lime water to milk.

The technique employed in this investigation is the same as that used by Van Slyke and Bosworth[2] in their studies of cow's

[1] Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 707.

[2] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 135. Bosworth, *ibid.*, 1915, xx, 707. Bosworth and Van Slyke, *ibid.*, 1916, xxiv, 177.

milk, human milk, and goat's milk. A complete chemical analysis of the milk being first obtained, it is then filtered through porous porcelain filters. The filtration divides it into two portions, a soluble filterable portion and an insoluble or unfilterable portion.

TABLE I.

Changes Produced by the Addition of Lime Water to Cow's Milk.

Sample No.	1		2			
Lime water added, per cent.	0	50	0	16⅔	33⅓	50
Reaction (+ = acidity; − = alkalinity) as cc. of 0.1 N required to neutralize 100 cc.						
Whole milk	+6.8	−15.3	+8.4	0.0	−7.0	−16.6
Serum	+6.6	+ 1.9	+7.8	+4.2	+2.2	+ 2.2
CaO*	gm.	gm.	gm.	gm.	gm.	gm.
Total	0.1947	0.2982	0.2106	0.2505	0.2710	0.3141
Insoluble combined with casein	0.0759	0.0759	0.0857	0.0857	0.0857	0.0857
Insoluble combined with P_2O_5	0.0524	0.1750	0.0586	0.0934	0.1291	0.1738
Total insoluble	0.1283	0.2509	0.1443	0.1891	0.2148	0.2595
Soluble	0.0664	0.0473	0.0663	0.0614	0.0562	0.0546
P_2O_5*						
Total	0.2314	0.2314	0.2554	0.2554	0.2554	0.2554
Organic in casein	0.0497	0.0497	0.0545	0.0545	0.0545	0.0545
Inorganic, total	0.1817	0.1817	0.2009	0.2009	0.2009	0.2009
" insoluble	0.0633	0.1433	0.0752	0.1172	0.1376	0.1595
" soluble	0.1154	0.0284	0.1257	0.0837	0.0633	0.0414
Citric acid.*						
Total	0.1684	0.1684	0.1795	0.1795	0.1795	0.1795
Insoluble	0.0000	0.0381	0.0000	0.0000	0.0184	0.0370
Soluble	0.1684	0.1301	0.1795	0.1795	0.1611	0.1425
Casein	3.10	3.10	3.40	3.40	3.40	3.40

* Per 100 cc. of original milk.

The composition of these two portions was determined by chemical analysis.

Cow's milk when used as a food for infants is usually diluted with an equal or greater volume of water which may carry other

substances in solution or suspension, such as lactose, calcium hydroxide, barley flour, maltose, etc., and in order to make the conditions surrounding the milk used for this investigation as nearly comparable to feeding conditions as possible the desired amount of lime water was added to the milk, and this mixture then diluted to twice the volume of the original milk. The results obtained by our study, however, have been reported on the basis of the original volume of the milk.

Our investigation shows that while the addition of lime water to milk increases the total CaO present it brings about a marked change in the arrangement of the salts, which results in a pre-

TABLE II.

Insoluble Acids and Bases in Milk and Milk Plus Lime Water Calculated to Gm. Equivalents.

Sample No.	Lime water added.	Casein as an octavalent acid.	$\frac{P_2O_5}{2}$ as a divalent acid.	Citric acid as trivalent acid.	Sum of acids.	Calcium.	Excess of base.
	per cent						
1	0	27.9×10^{-4}	17.8×10^{-4}	0.0×10^{-4}	45.7×10^{-4}	45.8×10^{-4}	0.1×10^{-4}
1	50	27.9	40.4	6.0	74.3	89.6	15.3
2	0	30.6	21.2	0.0	51.8	51.5	0.0
2	$16\frac{2}{3}$	30.6	33.0	0.0	63.6	67.5	3.9
2	$33\frac{1}{3}$	30.6	38.8	2.9	72.3	76.7	4.4
2	50	30.6	44.9	5.8	81.3	92.7	11.4

cipitation of calcium, phosphorus, and citric acid. The figures in Table I show that by the addition to milk of 50 per cent of lime water the soluble CaO is reduced to less than one-half of the amount present in the original milk, the soluble phosphorus to less than a third, and the soluble citric acid to about three-fourths.

The amounts of the insoluble constituents present in the milk and the milk to which the lime water has been added as shown in Table I have been calculated to gm. equivalents and will be found in Table II. In making these calculations phosphoric acid has been considered a divalent acid because it has been shown that the insoluble inorganic phosphorus normally present in fresh cow's milk is in the form of dicalcium phosphate, $CaHPO_4$.[3] It

[3] Van Slyke and Bosworth, *J. Biol. Chem.*, 1915, xx, 135.

will be noticed that in the fresh milk there is a balance of bases and acids, while the addition of lime water to the milk produces an excess of base, which means of course, that the insoluble phosphate is now a mixture of di- and tricalcium phosphate.

The acidity or alkalinity as determined by the method of Van Slyke and Bosworth[4] also demonstrates the presence of alkaline phosphates, for it will be noticed that while the original milk and its serum had an acid reaction, the addition of lime water brought the reaction of the serum towards the neutral point and the reaction of the unfiltered milk became alkaline, showing that in filtering the milk to obtain the serum, insoluble alkaline salts were removed or failed to pass through the filter. If milk to which 50 per cent of lime water has been added is centrifugalized, a sediment is obtained which is a mixture of calcium caseinate and calcium phosphates. Such a sediment obtained by us and treated with alcohol and ether to dry it gave the following figures upon analysis.

	per cent
Casein	21.84
Total P_2O_5	34.784
Organic P_2O_5 in casein	0.350
Inorganic P_2O_5	34.434
CaO	32.474

Upon calculating these figures to gm. equivalents, we obtain casein 19.7×10^{-3}, $\frac{P_2O_5}{2}$ inorganic as a divalent acid 969.8×10^{-3}, sum of casein and inorganic P_2O_5 989.5×10^{-3}, calcium oxide $1{,}159.8 \times 10^{-3}$. It will be seen from these figures that the calcium present is more than enough to form the neutral dicalcium phosphate with the P_2O_5 present, but not enough to form the alkaline tricalcium phosphate, the result being a mixture of the two.

In this connection it is of interest to recall that Clark[5] in studying the change in hydrogen ion concentration produced by the addition of lime water, or sodium citrate to milk concluded that the use of sodium citrate was the more objectionable because it produced an alkaline reaction in the milk, while the use of

[4] Van Slyke and Bosworth, *J. Biol. Chem.*, 1914, xix. 73.

[5] Clark, W. M., *J. Med. Research*, 1914–15, xxxi. 431.

lime water produces a reaction near the neutral point. As he used the electrical method to determine his hydrogen ion concentrations it will be seen at once that he failed to take into consideration the insoluble alkaline phosphates present.

CONCLUSIONS.

The addition of lime water to milk, which normally contains some insoluble dicalcium phosphate, results in the precipitation of more calcium phosphate, the insoluble phosphates under these conditions being a mixture of di- and tricalcium phosphate, $CaHPO_4$ and $Ca_3P_2O_8$.

The addition of lime water to milk brings the reaction of the serum towards the neutral point, the soluble alkalinity of the lime water being used up in the precipitation of the insoluble calcium phosphates mentioned above.

When milk to be used for infant feeding is treated with lime water and finally diluted to such an extent that it has twice the volume of the original milk, or more, the soluble calcium and phosphorus may be reduced to amounts less than those which are present in human milk.

A SAPONIN FROM YUCCA FILAMENTOSA.*

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(Received for publication, November 27, 1916.)

The presence of a saponin in *Yucca filamentosa* was first reported by Morris[1] who did not analyze his preparation. Schulz[2] analyzed a preparation isolated by Meyer[3] and found 58.61 per cent of carbon and 8.31 per cent of hydrogen. He calculated the formula of the saponin to be $C_{24}H_{40}O_{10}$ or $C_{40}H_{68}O_{17}$. The theoretical percentages of these formulas are respectively C 58.02, H 8.20, O 32.78, and C 58.56, H 8.29, O 33.15. He did not determine the molecular weight of the compound. Kobert[4] considers that the formula agreeing best with the analysis of Schulz is $C_{67}H_{118}O_{28}$, which requires C 58.64, H 8.68, O 32.68 per cent, and a molecular weight of 1,371. Schulz states that the saponin was not soluble in water.

We have isolated an amorphous, ash-free saponin from the rootstock of *Yucca filamentosa* which was obtained from the vicinity of Biltmore, N. C., during the month of March. This saponin was soluble in water and its ultimate composition was different from the saponin described by Schulz.

The analyses average C 52.16, H 7.17, and O 40.67 per cent. The molecular weight determined in phenol was found to be 537. These figures agree closely with the formula $C_{24}H_{40}O_{14}$, which corresponds with the general formula for saponins proposed by Kobert,[5] $C_nH_{2n-16}O_{28}$, if this is divided by two.

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[1] Morris, A. M., *J. Pharm.*, 1895, 520.

[2] Schulz, V. W., *Arbeit. pharm. Inst. Dorpat*, 1896, xiv, 110.

[3] Meyer, A., see Schulz.[2] Schulz refers to the plant as *Yucca filamenta*. This is evidently an older name for *Yucca filamentosa*.

[4] Kobert, R., *Abderhalden's Biochem. Handlexikon*, Berlin, 1912, vii, 224.

[5] Kobert, *Unna Festschrift*, Leipsic, 1910, i, 161.

On hydrolysis, the saponin yielded a crystalline sapogenin melting at 175°C., also glucose and apparently glucuronic acid.

We have not been able to isolate any saponin from *Yucca filamentosa* which agrees with that reported by Schulz. It is possible that saponins from the same species of plants may differ, depending on the place where grown and the time of year when picked. We have some evidence on this point from work done recently on other species of plants.

EXPERIMENTAL.

Preparation of the Saponin.—The rootstocks of *Yucca filamentosa* were cut into slices and dried at 50°C. in air desiccated over calcium chloride. The dried substance was ground to a coarse powder in a mill. 5 kilos of the substance were extracted five times with hot 95 per cent alcohol by heating in a steam kettle. The alcoholic extract was concentrated to a small volume in a vacuum still, 200 gm. of magnesium oxide were added, and the mixture was evaporated to dryness on the steam bath. The residue was pulverized and extracted with hot absolute alcohol. On standing, the saponin was deposited as a light brown amorphous substance. This was filtered off and washed with absolute ether, the yield of crude saponin being about 6 per cent. It was purified by dissolving in hot absolute alcohol, cooling the solution, filtering, and finally washing the saponin with absolute ether. To obtain the saponin colorless and free from ash, it was necessary to repeat this process several times. On pouring the mother liquors into an equal volume of absolute ether, more saponin was obtained. The supernatant liquid was decanted and the precipitates were purified as stated above.

The aqueous solution of the saponin was neutral to litmus and had the foaming properties characteristic of most saponins. Lead acetate, lead subacetate, and barium hydroxide did not precipitate it from the dilute or concentrated aqueous solution. A cholesterol compound could not be made. When evaporated with sulfuric acid it gave a deep red color. The saponin was very hygroscopic and, for analysis, was first dried in a vacuum oven by gradually heating to 105°C. It was then dried over night in an electric oven at 110°C.

Analyses of four different preparations gave the following results.

I.	0.2009	gm.	substance	gave	0.3838	gm.	CO_2	and	0.1295	gm.	H_2O.
II.	0.2471	"	"	"	0.4731	"	"	"	0.1602	"	"
III.	0.1962	"	"	"	0.3749	"	"	"	0.1245	"	"
IV.	0.2608	"	"	"	0.4992	"	"	"	0.1657	"	"

	I.	II.	III.	IV.	Average.
C	52.10	52.21	52.12	52.20	52.16
H	7.21	7.25	7.10	7.11	7.17
O	40.69	40.54	40.78	40.69	40.67

0.3609 gm. substance lowered the freezing point of 34.29 gm. of phenol 0.147°.

$$M = \frac{7{,}500 \times 0.3609}{34.29 \times 0.147} = 537$$

	Calculated for $C_{24}H_{40}O_{14}$:	Found:
C	52.17	52.16
H	7.25	7.17
Mol. wt.	552	537

Hemolytic Action and Surface Tension of the Saponin.[6]—Blood was drawn from the heart of a Belgian hare into 0.9 per cent salt solution, containing a little potassium oxalate. The mixture was then centrifugated and was washed several times with Locke's[7] solution. Two drops of the residual mass of corpuscles were then added to 10 cc. of the aqueous solution of the saponin, 1 in 20,000, and this was kept at 37°C. Hemolysis took place in about 15 minutes. For the surface tension determination, 100 mg. of the saponin were dissolved in 1 liter of Locke's solution. The surface tension at 37°C., as determined by the Morgan drop weight method, was 56.69 dynes per cm.

Hydrolysis of the Saponin.

Preparation of the Sapogenin.—10 gm. of the saponin were dissolved in 100 cc. of 4 per cent sulfuric acid and the solution was heated for 7 hours on the steam bath. A tan-colored amorphous substance separated. The mixture was centrifugated and the substance washed with water until no longer acid to litmus. It was then dried over the steam bath and placed over sulfuric acid in a vacuum desiccator, where it became red and

[6] These determinations were made by Dr. H. E. Woodward of the Bureau of Chemistry.

[7] Locke's solution:

	gm.
NaCl	9.29
KCl	0.42
$CaCl_2$	0.24
$NaHCO_3$	0.15

H_2O to 1 liter.

horny in appearance. The yield was 4.9 gm., or about 50 per cent.

To complete the hydrolysis this sapogenin was then boiled with 50 cc. of 6 per cent sulfuric acid for 7 hours. At first it became soft and oily, but after about 5 hours it changed to a dark hard lump. The sulfuric acid then was poured off and the mass ground up and washed with water till the washings were no longer acid to litmus. The solid material dissolved completely in acetone and was easily crystallized from dilute acetone or dilute alcohol. The fine, acicular crystals melted at 175°C. The alcoholic solution of this sapogenin was not precipitated by alcoholic lead acetate or basic lead acetate. No change was observed on adding an alcoholic solution of ferric chloride. It was insoluble in 10 per cent alkali and acids. Glacial acetic acid dissolved it readily.

The molecular weight determined in phenol was 255. 0.1004 gm. of substance lowered the freezing point of 26.17 gm. of phenol 0.113°.

$$M = \frac{7{,}500 \times 0.1004}{26.17 \times 0.113} = 255$$

Identification of Glucose.—The acid solutions obtained by the hydrolysis of the saponin were neutralized with barium carbonate, filtered, clarified with animal charcoal, and evaporated on the steam bath, thus giving a yellow syrup. On cooling, this syrup solidified. We obtained a phenyl osazone melting at 205°C. and a bromophenyl osazone melting at 220°C. We did not succeed in preparing a hydrazone, indicating the absence of mannose. The absence of mannose, together with the dextrorotation of the aqueous solution of the syrup, and the melting points of the osazones show that this sugar is glucose.

Tests Indicating the Presence of Glucuronic Acid.—In addition to glucose, the sugar syrup obtained by hydrolysis of the saponin also contained a substance which gave furfural on distillation with 12.5 per cent hydrochloric acid. A pentose phenyl or bromophenyl hydrazone or osazone could not be prepared, and Bial's[8] reagent (orcin, hydrochloric acid, and a trace of ferric chloride) failed to give a positive test. It is therefore unlikely that a pentose was present.

[8] Bial, M., *Biochem. Z.*, 1907, iii, 323.

When the syrup was heated with naphthoresorcinol and hydrochloric acid, and the cooled mixture shaken with ether, the ether solution was colored a beautiful purple. The reaction with naphthoresorcinol and the formation of furfural together with the failure of Bial's reagent to give a positive test are strong indications of the presence of glucuronic acid. The close resemblance of the phenyl and bromophenyl osazone of glucose and glucuronic acid makes it difficult to identify the latter in a mixture of the two.

The presence of glucuronic acid as a product of hydrolysis in substances obtained from plants has been reported in a few cases, and Asahina and Momoya[9] have established its presence in the saponin of *Styrax japonica*.

Lack of material prevented further work.

Location of the Saponin in the Tissue.

The saponin has been definitely located in the fibrovascular bundles of the roots and the leaf bases. Since the plant belongs to the monocotyledons, a great number of fibrovascular bundles occur in the roots. The number of bundles roughly estimated in a root (1 cm. thick) was considerably over 1,000. The saponin was found within the tracheæ. In almost every bundle a trachea cell was observed, which was partially or wholly filled with an amorphous mass, more or less transparent. The color of these masses was orange-brown in the roots and brownish red in the bases of the leaves. Saponin masses were not found in parenchyma cells of the wood or bark. This observation is especially interesting since, as far as a study of the literature shows, this is the first time that saponin has been found in the tracheæ.

The coloration in the tracheæ obtained with sulfuric acid was first distinctly yellow, followed by a very slow change to pink and reddish violet. After standing several hours the color became dark brown and the tissue was largely destroyed. Occasionally deep brownish red globule-like bodies were observed in or close to the tracheæ.

When acetic anhydride was added to the section before the

[9] Asahina, Y., and Momoya, M., *Arch. pharm.*, 1914, cclii, 56.

addition of sulfuric acid or sulfuric acid-alcohol, the color change to pink or reddish violet could be observed almost immediately. Slight heating of the section with sulfuric acid-alcohol also had the same effect in the coloring of the tracheæ bundles. Addition of a trace of 10 per cent ferric chloride solution to the section freshly mounted in sulfuric acid-alcohol produced a decided green color. Precipitates were not observed.

Since experiments with the isolated saponin had shown that it could not be precipitated by neutral or basic lead acetate, barium hydroxide, or cholesterol, it was thought probable that the hemolytic properties of the saponin might be made use of in supporting the previous evidence as to the location of the saponin in the tissue. For this purpose blood from the heart of a rabbit was obtained and, after defibrinizing or adding sodium oxalate to prevent the precipitation of fibrin, the blood corpuscles were washed with physiological sodium chloride and Locke's solution. The blood corpuscles obtained by centrifugating were directly applied in a suspension of about 1:1 Locke's solution to the dry section. The turbid suspension infiltrated into the tissue which became invisible under the microscope, but in a few seconds certain parts of the tissue cleared up. The blood corpuscles disappeared, the liquid became transparent and discolored, and the tissue clearly visible. Repeated observations showed that the tracheæ cells always were visible first and the masses in these consequently had the strongest hemolytic action. Crystals of the sapogenin obtained as previously described were tested with blood in the same manner under the microscope, but practically no hemolytic action was observed.

Briefly stated, the saponin masses present in the tracheæ had the same properties as the saponin isolated from the plant. They were easily soluble in water and also in hot alcohol (95 per cent), and insoluble in benzene or chloroform. They showed the characteristic color reactions with concentrated sulfuric acid, sulfuric acid-alcohol (1:1), sulfuric acid with the addition of a trace of a 10 per cent ferric chloride solution, or acetic anhydride followed by concentrated sulfuric acid. The masses had a strong hemolytic action.

The microchemical experiments were carried out on fresh material collected in the fall, on the same material dried at room

temperature, and also on the same material dried for several hours at 100°C. Cross and longitudinal sections were prepared and treated either directly with the reagents or after extraction with ether to remove fatty and coloring substances.

While in material dried at 100°C. the saponin masses covered the cell walls of the tracheæ like a film, often not easily visible, these masses were very conspicuous in material dried at room temperature.

SUMMARY.

1. A new saponin, $C_{24}H_{40}O_{14}$, was isolated from the rootstock of *Yucca filamentosa*. The yield of crude saponin was about 6 per cent. It was soluble in water, alcohol, phenol, and glacial acetic acid, and could not be precipitated from the aqueous solution by neutral lead acetate, basic lead acetate, and barium hydroxide. No cholesterol compound could be prepared. Hemolysis was observed after 15 minutes in the saponin solution (1 to 20,000) containing rabbit blood, and kept at 37°C. The surface tension at 37°C. was 56.69 dynes per cm.

2. Hydrolysis yielded a sapogenin, crystallizing in regular, fine needles melting at 175°C. The molecular weight of this sapogenin was approximately 255. It was soluble in alcohol, phenol, and glacial acetic acid, but was not soluble in either alkali or dilute acids. Alcoholic solutions of lead acetate, basic lead acetate, and ferric chloride had no effect on the alcoholic solution of this substance. The crystals tested under the microscope had practically no hemolytic action on blood corpuscles.

3. Hydrolysis of this saponin also yielded a sugar identified as glucose. Evidence was obtained which indicates that glucuronic acid is formed when the saponin is hydrolyzed.

4. The saponin is located as brownish amorphous masses in the fibrovascular bundles of the roots and leaf bases.

A NEW METHOD OF STEAM DISTILLATION FOR THE DETERMINATION OF THE VOLATILE FATTY ACIDS, INCLUDING A SERIES OF COLORIMETRIC QUALITATIVE REACTIONS FOR THEIR IDENTIFICATION.*

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INTRODUCTION.

In the course of an investigation in this laboratory it became necessary to have at hand a simple and convenient method for the identification and quantitative determination of the volatile saturated fatty acids, and to that end the investigation here reported was undertaken.

The imperfections of the well known Duclaux method for the determination of these acids are generally recognized among chemists who have occasion to apply it. When mixtures of acids are distilled identification by the distillation numbers alone is somewhat uncertain, a fact rather lightly touched upon

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by Duclaux himself.[1] Calculating results from the distillation numbers is a tedious process and, such being the case, appreciable errors may be introduced.

The method about to be described for the identification and determination of the volatile fatty acids is one of steam distillation rather than direct distillation. By the use of this method of steam distillation the vaporization rate of the individual acids, when plotted on a simple logarithmic chart, appear as straight lines. On the other hand, the vaporization rate of mixtu es of acids will be depicted on the same chart as curved lines. For purposes of identification, therefore, the results of the steam distillation may be interpreted by direct comparison with the chart instead of with tables as in the Duclaux method. In addition, there is given a series of colorimetric qualitative tests, depending upon the difference in solubility of the iron and copper salts of the acids in various organic solvents, which serve to confirm the results of the steam distillation. The calculations in the method herein described are of the simplest nature and are doubtless familiar to every chemist.

Attention is called to an article by Stein[2] in 1913, which seems to have passed unnoticed as far as can be judged from the recent literature. Stein states: If dilute aqueous solutions of substances volatile with steam are distilled under conditions of constant pressure, temperature, and volume in the distilling flask, the process proceeds with a certain regularity. Stein gives the credit for this discovery to Naumann and Müller.[3]

Stein carried out distillations with twenty organic acids, seven of which consisted of the first members of the volatile fatty acids. In most cases he made these distillations while keeping the volume of the liquid in the distilling flask at 150 cc. He constructed a table giving the percentage amount of each acid distilling over into the first 100 cc. fraction of the

---

[1] Duclaux, ". . . . . en cherchant sur les tables si la marche des nombres trouvés par l'expérience coïncide avec une des séries calculées. Cette coïncidence n'est naturellement jamais parfaite: les incertitudes de la méthode des distillations, les irrégularités inévitables dans la mesure des prises et dans les dosages ne le permettent pas." Duclaux, Dosage des principaux produits de la fermentation alcoolique, *Traité de Microbiologie*, 1900, iii, 394.

[2] Stein, A., Gesetzmässigkeiten bei der Wasserdampfdestillation organischer Säuren, *J. prakt. Chem.*, 1913, lxxxviii, 83.

[3] Naumann, A., and Müller, W., Regelmässigkeiten beim Destilliren verdünnter wässriger Phenollösungen, *Ber. chem. Ges.*, 1901, xxxiv, 224.

distillate while the volume in the distilling flask was maintained at 150 cc. His results are recorded in Table I.

Some of the percentages obtained by Stein from duplicate determinations made on the same acid do not appear to agree very closely. There is no description of the steam distilling apparatus used in determining these percentages and it is possible that the one made use of was somewhat unsuited to the purpose. The distillations were, therefore, repeated in this laboratory, the same acids being used to redetermine the percentage amounts. In addition, there have been added to the list the percentage amounts of acid distilling over into the first 100 cc. fraction for isocaproic, heptylic, caprylic, and pelargonic acids. Of the three remaining higher acids, capric acid is soluble only to a very slight extent, undecylic acid was not available, and lauric acid is practically insoluble. Therefore these three acids were not included in this investigation. The figures obtained with the acids investigated will be found in Table I under those obtained by Stein.

TABLE I.

Comparative Percentages of Acid Which Distil Over into the First 100 Cc. Fraction of Distillate from a Constant Volume of 150 Cc. as Found by Stein and by Dyer.

Acid.	Formic.	Acetic.	Propionic.	*n*-Butyric.	Isobutyric.	Valeric.**	*n*-Caproic.	Isocaproic.	Heptylic.	Caprylic.	Pelargonic.
Stein.	20.98 22.02	33.09	55.21 58.51	70.53	79.3 80.35 80.85	87.7	91 94				
Dyer.	17.89*	30.75*	52.67*	69.88	79.73	87.51	89.75	92.60	94.52	98.45	100.00

* Average of determinations as noted below in Table II.

** Stein's valeric acid was evidently the iso acid since it had the same distilling constant as the isovaleric acid used in this laboratory.

The figures for the first three acids mentioned represent the average percentages from several determinations as shown in Table II. The values found for formic, acetic, and propionic acids are somewhat lower than those obtained by Stein; in the other cases the figures agree fairly well.

The results so obtained indicated that a practical use might be made of the "distilling constants" for the identification and determination of those fatty acids volatile with steam. As already noted, the second 100 cc. fraction of the distillate had

TABLE II.

Amount of Each Acid Taken for the Distillation Together with the Amount of That Acid Distilling Over into the First and (in Some Cases) Second 100 Cc. Fractions of Distillate as Determined by Titration with 0.1 N KOH.

Acid.	Total amount of acid distilled.	Amount of acid present in 1st 100 cc. fraction.		Amount of acid present in 2nd 100 cc. fraction.		Calculated amount of acid in 2nd 100 cc. fraction.‡
	gm.	*gm.*	*per cent*	*gm.*	*per cent*	*per cent*
Formic.						
Sample 1*...	0.49413	0.08882	17.97	0.07732	15.65	14.69
" 2*...	0.59873	0.10585	17.68	0.08974	14.99	14.55
" 2....	0.58962	0.10623	18.01	0.09411	15.96	14.77
Average.....			17.89		15.53	14.67
Acetic.						
Sample 1*...	0.22192	0.06821	30.74	0.04863	21.91	21.29
" 1....	0.41008**	0.12573	30.66			
" 1....	0.41075**	0.13362	30.74			
" 1....	0.41111	0.12621	30.70			
" 1....	0.41267†	0.12849	31.14			
" 1....	0.62708	0.19496	31.09	0.13696	21.86	21.04
" 2*...	0.41033	0.12447	30.34	0.08868	21.61	21.13
" 2....	0.41333	0.12609	30.51	0.09162	22.16	21.20
" 3*...	0.41891	0.12915	30.83	0.09186	21.17	21.33
Average.....			30.75		21.74	21.20
Propionic.						
Sample 1*...	0.47637	0.24974	52.43	0.11850	24.88	24.94
" 1....	0.48622	0.25774	53.01	0.12332	25.36	24.91
" 1....	0.98220	0.51629	52.56	0.23708	24.14	24.93
Average.....			52.67		24.79	24.93

*Sample 1 of formic acid, Eimer and Amend, "from Germany."
" 2 " " " Baker and Adamson, "100 per cent."
" 1 " acetic " old sample of glacial acid, source unknown.
" 2 " " " J. T. Baker, "99.5 per cent."
" 3 " " " source unknown, "95.00 per cent."
" 1 " propionic acid, Kahlbaum.

** Distillate collected in 10 cc. fractions.

† Distillate collected in an ordinary 100 cc. graduate. Other distillates were collected in various 100 cc. graduated flasks.

‡ The figures here given were obtained as described under Experimental.

TABLE III.

Titrations. Cc. of 0.1 N KOH Required for Each Successive 10 Cc. Fraction of Distillate up to 100 Cc.

Acid Cc. of 0.1 N KOH Equivalent gm. of acid.		Formic. 216.05 0.9943	Acetic. 171.52 1.0298	Propionic. 132.64 0.9822	*n*-Butyric. 106.52 0.9383	Isobutyric. 107.19 0.9443	Isovaleric. 91.45 0.9338	*n*-Caproic. 8.19 0.0951	Isocaproic. 8.93 0.1037	Heptylic. 14.26 0.1856	Caprylic. 6.44 0.0928	Pelargonic. 2.82 0.0446
	cc.											
1	10	4.40	6.11	9.33	12.22	16.74	17.40	1.85	1.92	3.43	2.18	0.80
2	10	4.28	6.04	8.70	10.88	13.88	13.78	1.35	1.56	2.67	1.27	0.60
3	10	4.30	5.87	8.12	9.44	12.20	11.32	1.00	1.20	2.12	0.78	0.38
4	10	4.20	5.56	7.53	8.78	10.37	9.10	0.82	0.98	1.60	0.65	0.22
5	10	4.18	5.44	7.09	7.48	8.48	7.60	0.62	0.76	1.28	0.50	0.20
6	10	4.17	5.13	6.64	6.52	7.46	6.08	0.49	0.56	0.77	0.30	0.10
7	10	4.07	4.94	6.23	5.65	6.13	5.10	0.40	0.43	0.55	0.20	0.12
8	10	4.02	4.73	5.87	5.02	5.13	3.94	0.31	0.35	0.40	0.14	0.10
9	10	3.99	4.48	5.35	4.48	4.20	3.07	0.28	0.28	0.34	0.10	0.10
10	10	3.75	4.31	4.85	3.98	3.43	2.53	0.23	0.23	0.32	0.10	0.10
Total 1st 100 cc.		41.36	52.61	69.71	74.45	88.02	79.92	7.35	8.27	13.48	6.22	2.72*

* See note to same acid in Table IV.

TABLE IV.

Actual Percentage of Acid Distilling Over into Any Given Fraction.

Fraction.	Formic.	Acetic.	Propionic.	*n*-Butyric.	Isobutyric.	Isovaleric.	Isocaproic.	*n*-Caproic.	Heptylic.	Caprylic.	Pelargonic.
cc.											
10	2.03	3.56	7.03	11.47	15.62	19.03	21.50	22.59	24.05	33.85	
20	4.01	7.08	13.59	21.68	28.57	34.10	38.97	39.07	42.77	53.57	
30	6.00	10.50	19.71	30.54	39.95	46.48	52.41	51.28	57.64	65.68	
40	7.94	13.74	25.39	38.78	49.62	56.43	63.38	61.29	68.86	75.77	
50	9.87	16.91	30.74	45.80	57.53	64.74	71.89	68.86	77.84	83.53	
60	11.80	19.90	35.75	51.92	64.49	71.39	78.16	74.84	83.24	88.19	
70	13.68	22.78	40.45	57.22	70.21	76.97	82.98	79.73	87.10	91.30	
80	15.54	25.54	44.88	61.93	75.00	81.28	86.90	83.52	89.90	93.48	
90	17.39	28.15	48.91	66.14	78.92	84.64	90.02	86.94	92.28	95.03	
100*	18.50	30.66	52.56	69.88	82.12	87.51	92.60	89.75	94.52	96.58	100.00**
200	35.5	50.83	76.31	90.53	85.69	97.17		98.95	99.43		
300	46.5	64.85	87.06	96.05	98.40	98.45		100.89			
400	56.0	75.50	92.29	97.68		98.75					
500	63.5		95.16	98.22							
600	69.8		96.44	98.45							
700	74.8		97.04								
800	79.2		97.41								
900	83.2										
1,000	86.3										
1,100	89.0										
1,200	91.0										
1,300	92.8										
1,400	93.8										
1,500	95.0										
1,600	95.8										
1,700	96.6										
1,800	97.0										
1,900	97.9										
2,000	98.1										

* Footnotes on following page.

been collected in some cases and it was observed that the amount of acid in this second fraction bore a certain ratio to the amount of acid in the first 100 cc. fraction.

EXPERIMENTAL.

General Procedure and Analytical Results.

The method of procedure followed in determining these distilling constants was as follows: First, the amount of acid taken for the distillation was titrated with 0.1 N KOH using phenolphthalein as indicator. The acid was then liberated with an equivalent amount of 0.1 N H_2SO_4 and the steam distillation was carried out while the acid solution in the distilling flask was kept at a constant volume of 150 cc. Finally, the first 100 cc. fraction of the distillate was titrated with the 0.1 N KOH. The number of cc. of 0.1 N KOH so required was then divided by the number of cc. of 0.1 N KOH required for the total acid, the result multiplied by 100, giving the percentage amount of that acid distilling over into the first 100 cc. fraction. In like manner the percentage amount of acid distilling into the second 100 cc. fraction was obtained. It was noticed that upon subtracting the percentage amount of acid distilling over into the first 100 cc. fraction from 100 per cent, and multiplying the remainder by the same percentage figure followed by its division by 100, the result was practically equal to the percentage of acid obtained by the direct titration of the second 100 cc. fraction (see last column of Table II).

---

* The percentages of formic acid in succeeding fractions were determined on another portion of acid, the distillates from which were collected in 100 cc. fractions. In the case of the other acids the data indicated for fractions above 100 cc. were calculated from figures not given in the previous table; they are included merely to extend the columns somewhat further.

** The figures in Table III, were obtained by taking 0.2 cc. of pelargonic acid, adding it to about 100 cc. of water, and titrating with 0.1 N KOH. It was found that 11.27 cc. were required. The potassium salt solution of the acid was made up to a volume of 200 cc., from which a 50 cc. aliquot (equivalent to 2.82 cc. of 0.1 N KOH = 0.0446 gm. of acid) was taken for the distillation. The requisite amount of 0.1 N H_2SO_4 was added, and the distillate was collected in 10 cc. fractions. It is impossible to calculate even an approximately correct distilling constant for pelargonic acid from these data. Therefore, a second 50 cc. aliquot was distilled as before but the distillate was collected in a 100 cc. graduated flask, the contents of which required 2.72 cc. of 0.1 N KOH. One drop of the alkali sufficed to color the second 100 cc. fraction a decided pink. It is concluded, therefore, that the distilling constant for pelargonic acid is practically 100, and it has been so drawn on the logarithmic chart.

TABLE V.

Calculated Percentage of Acid Distilling Over into Any Given Fraction.

Fraction.	Formic.	Acetic.	Propionic.	*n*-Butyric.	Isobutyric.	Isovaleric.	Isocaproic.	*n*-Caproic.	Heptylic.	Caprylic.	Pelargonic.
cc.											
10	2.03	3.56	7.03	11.47	15.62	19.03	21.50	22.59	24.05	33.85	
20	4.02	6.99	13.57	21.62	28.80	34.44	38.38	40.08	42.32	56.24	
30	5.97	10.30	19.65	30.61	39.92	46.92	51.63	53.62	56.19	71.05	
40	7.88	13.49	25.30	38.57	49.30	57.02	62.03	64.10	66.73	80.85	
50	9.75	16.57	30.55	45.62	57.22	65.20	70.19	72.21	74.73	87.33	
60	11.58	19.54	35.43	51.86	63.90	71.82	76.60	78.49	80.81	91.62	
70	13.37	22.37	39.97	57.38	69.54	77.18	81.63	83.35	85.43	94.46	
80	15.13	25.10	44.19	62.27	74.30	81.52	85.58	87.11	88.93	96.34	
90	16.85	27.73	48.11	66.60	78.31	85.04	88.68	90.02	91.59	97.58	
100	18.54	30.27	51.76	70.43	81.70	87.89	91.11	92.27	93.61	98.40	100.00
200	33.64	51.38	76.73	91.26	96.65	98.53	99.21	99.40	99.59	99.97	
300	45.94	66.10	88.77	97.42	99.39	99.82	99.93	99.95	99.97	100.00	
400	55.96	76.36	94.58	99.24	99.89	99.98	99.99	100.00	100.00		
500	64.13	83.52	97.39	99.78	99.98	100.00	100.00				
600	70.78	88.51	98.74	99.93	100.00						
700	76.20	91.99	99.39	99.98							
800	80.61	94.41	99.71	100.00							
900	84.20	96.10	99.86								
1,000	87.13	97.28	99.93								
1,100	89.52	98.10	99.97								
1,200	91.46	98.68	99.99								
1,300	93.04	99.08	100.00								
1,400	94.33	99.36									

1,500	95.38	99.55
1,600	96.24	99.69
1,700	96.94	99.78
1,800	97.51	99.85
1,900	97.97	99.90
2,000	98.35	99.93
2,100	98.66	99.95
2,200	98.91	99.97
2,300	99.11	99.98
2,400	99.28	99.99
2,500	99.41	100.00
2,600	99.52	
2,700	99.61	
2,800	99.68	
2,900	99.74	
3,000	99.79	
3,100	99.83	
3,200	99.86	
3,300	99.89	
3,400	99.91	
3,500	99.93	
3,600	99.94	
3,700	99.95	
3,800	99.96	
3,900	99.97	
4,000	99.98	
4,100	99.99	
4,200	100.00	

TABLE VI.

Calculated Percentage of Acid Distilling Over into Any Given 100 Cc. Fraction.

Fraction.	Formic.	Acetic.	Propionic.	*n*-Butyric.	Isobutyric.	Isovaleric.	*n*-Caproic.	Isocaproic.	Heptylic.	Caprylic.	Pelargonic.
cc.											
100	17.89	30.75	52.67	69.88	79.73	87.51	89.75	92.60	94.52	98.45	100.00
200	32.58	52.04	77.60	90.93	95.89	98.44	98.95	99.45	99.70	99.98	
300	44.64	66.79	89.40	97.27	99.17	99.81	99.89	99.96	99.98	100.00	
400	54.54	77.00	94.98	99.18	99.83	99.98	99.99	100.00	100.00		
500	62.67	84.07	97.62	99.75	99.97	100.00	100.00				
600	69.35	88.97	98.87	99.92	99.99						
700	74.83	92.36	99.47	99.98	100.00						
800	79.33	94.71	99.75	99.99							
900	83.03	96.34	99.88	100.00							
1,000	86.07	97.47	99.94								
1,100	88.56	98.25	99.97								
1,200	90.61	98.79	99.99								
1,300	92.29	99.16	100.00								
1,400	93.67	99.42									
1,500	94.80	99.60									
1,600	95.73	99.72									
1,700	96.49	99.81									
1,800	97.12	99.87									
1,900	97.64	99.91									
2,000	98.06	99.94									
2,100	98.41	99.96									
2,200	98.69	99.97									
2,300	98.92	99.98									

2,400	99.11	99.99							
2,500	99.27	100.00							
2,600	99.40								
2,700	99.51								
2,800	99.60								
2,900	99.67								
3,000	99.73								
3,100	99.78								
3,200	99.82								
3,300	99.85								
3,400	99.88								
3,500	99.90								
3,600	99.92								
3,700	99.93								
3,800	99.94								
3,900	99.95								
4,000	99.96								
4,100	99.97								
4,200	99.98								

However, to prove this point completely it was deemed necessary to redistil the acids, collect the distillate in successive 10 cc. fractions, and titrate each fraction with 0.1 N KOH. The results obtained are recorded in Table III.

Each of the titrations of the successive 10 cc. fractions was divided by the value obtained from the original titration of the total acid and the result multiplied by 100. The figure so obtained was added to the sum total of the titrations of all preceding 10 cc. fractions treated in the same manner. Table IV shows the results obtained by this process.

Now, conceding that the percentage figure for the first 10 cc. fraction is the distilling constant for each acid (or is that percentage of the residual acid which distils over in each successive 10 cc. fraction), it is possible to carry out some interesting calculations.

Propionic Acid.

		100.00 − 7.03 = 92.97
92.97 × 7.03 = 6.54*	7.03 + 6.54 = 13.57	92.97 − 6.54 = 86.43
86.43 × 7.03 = 6.08	13.57 + 6.08 = 19.65	86.43 − 6.08 = 80.35

etc.

* Actually, $\frac{92.97 \times 7.03}{100} = 6.54$. The use of the divisor, 100, has been omitted for the sake of brevity.

Carrying out this system of subtracting the distilling constant, as represented by the percentage figure for the first 10 cc. fraction of each acid, from 100, multiplying the percentage remaining by the distilling constant, and dividing by 100, subtracting the figure so obtained from the percentage left, multiplying, and so on (accompanied by the addition of each quotient to the sum total of those preceding), we should have for each acid a column of figures, as in Table V.

The titrations made on the first 100 cc. fractions of distillate must, of course, be considered as relatively more accurate than those made on the first 10 cc. fractions, especially in the case of the higher and less soluble members of the series. Therefore, Table VI has been inserted. As in Table V, the same principle of calculation was used, but the distilling constants for the 100 cc. fractions (or the percentage amount of each acid distilling over into the first 100 cc. fraction) as given in Table I were employed.

These are simple logarithmic functions and they may be expressed on a simple logarithmic chart, as in Fig. 1.

Since the lines on the chart were drawn in accordance with the data obtained by titration of the first 100 cc. fraction, it will be noted that the line indicating the amount of *n*-caproic acid present in the first 10 cc. fraction of distillate crosses the

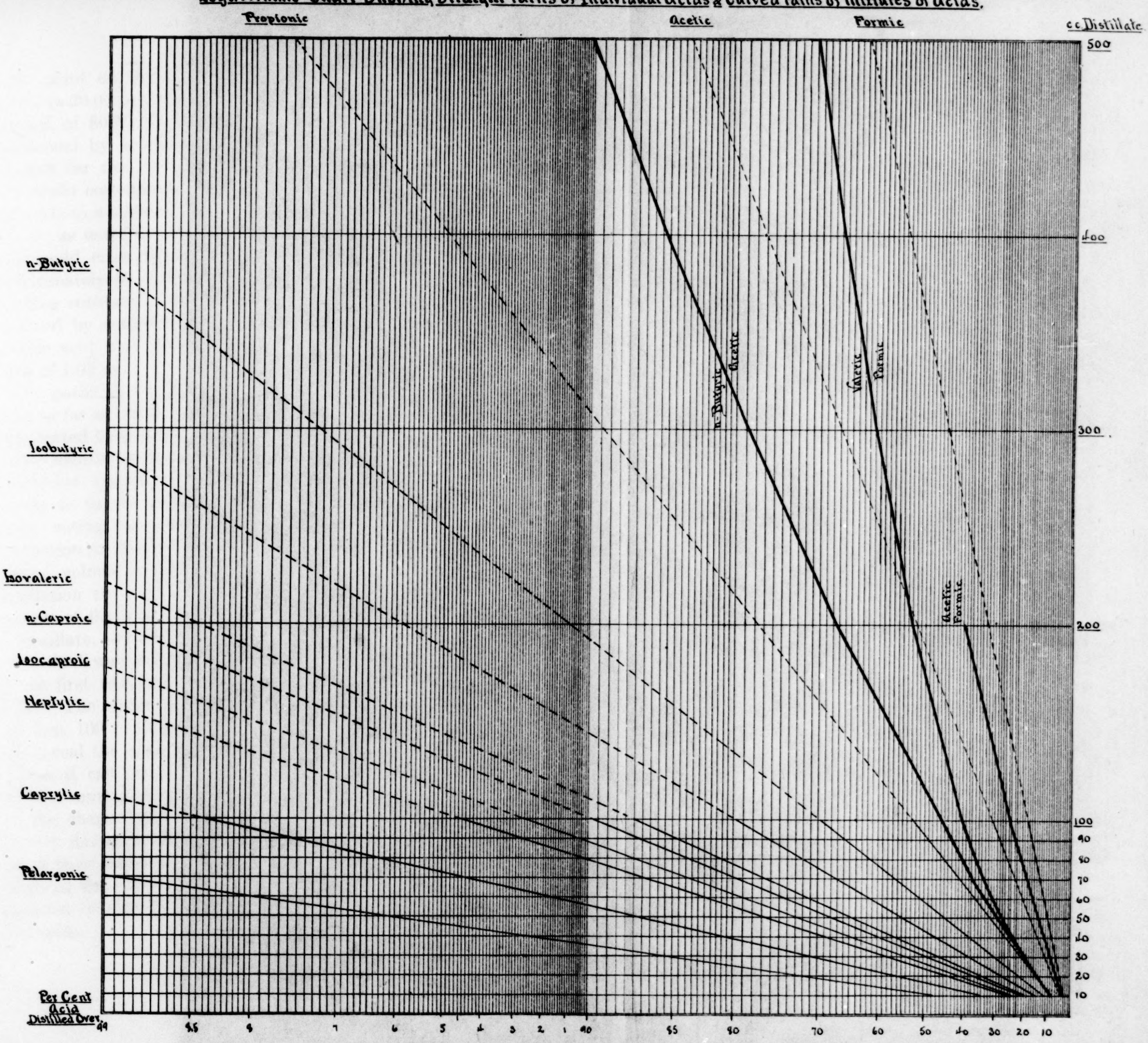
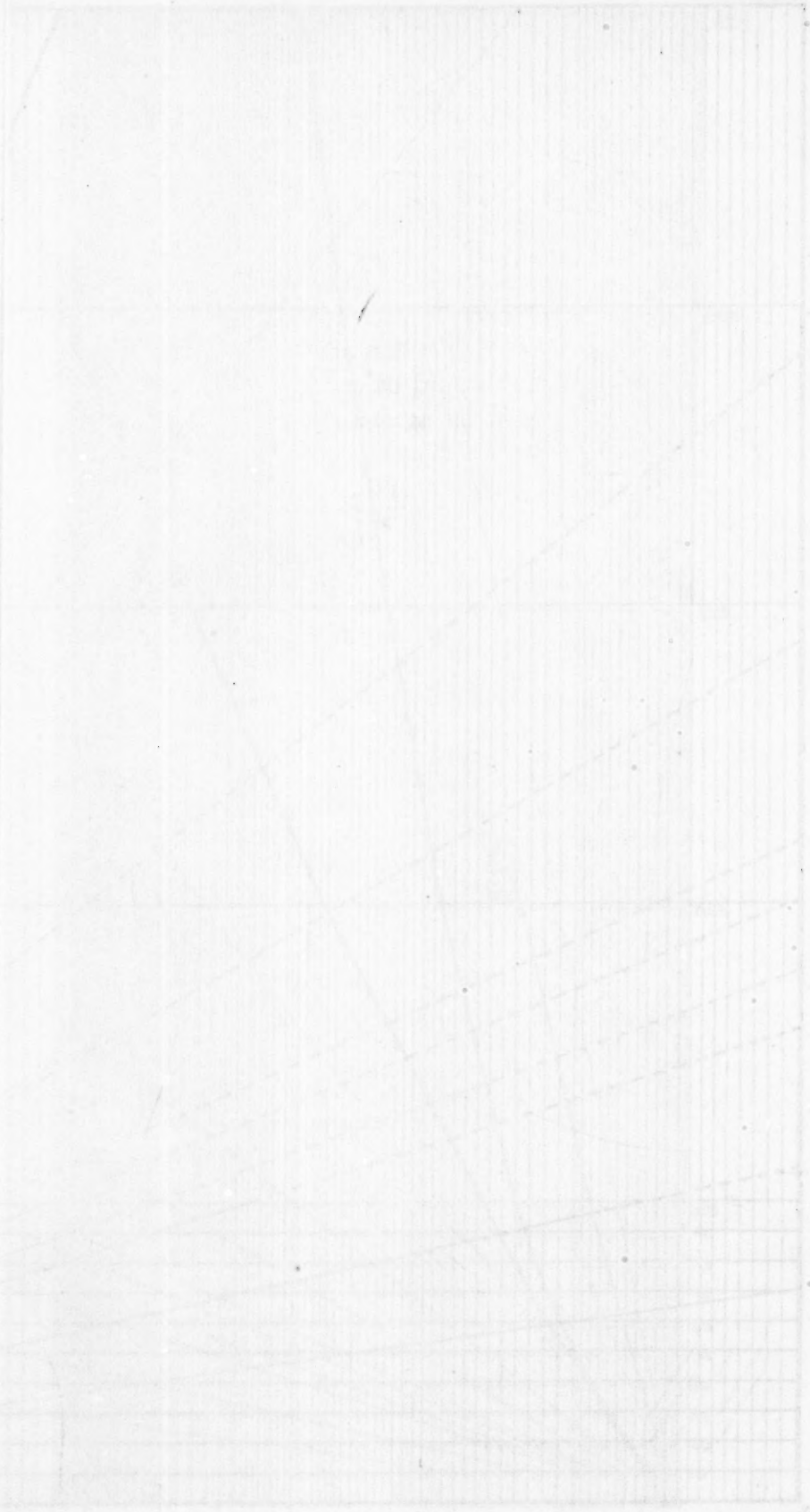


FIG. 1. Logarithmic chart.

(Dyer: Determination of Volatile Fatty Acids.)



10 cc. point on the chart at a number corresponding approximately to 20.00 per cent, which would calculate out to a distilling constant of 89.26 for the first 100 cc. fraction as against 89.75 determined by actual titration. A distilling constant of 20.00 per cent for the first 10 cc. fraction of distillate for *n*-caproic acid would correspond (in the case of the data in Table III for this acid) to a titration figure of 1.64 cc. of 0.1 N KOH instead of 1.85 cc. as noted in the table. Similarly, the line for isocaproic acid would cross the 10 cc. point at a position corresponding approximately to 23.00 per cent. This would calculate out to a distilling constant of 92.67 for this acid as against 92.60 as determined by actual titration. In Table III, under the caption for this acid, the titration should be 2.05 cc. of 0.1 N KOH instead of 1.92 cc.

The paths of the individual acids have been drawn on the chart as far as the first 100 cc. fraction of distillate, and continued with dotted lines as far as the fifth 100 cc. fraction.

The identification of a single acid by this method of steam distillation requires little or no explanation. It is necessary merely to titrate the total amount of acid present with 0.1 N alkali, noting the number of cc. required, and then arrange the distillation so that the amount of pure acid contained in the total 150 cc. volume in the distilling flask will be about 0.5 cc. For distillation the acid is, of course, liberated with an equivalent amount of 0.1 N or 0.2 N sulfuric acid. Any convenient amount of distillate, say 100 cc., is collected. This is titrated with the 0.1 N alkali and the figure so obtained is divided by that obtained in the first titration representing the total amount of acid distilled. The result will be the distilling constant of the acid for the first 100 cc. fraction of distillate, and a look at the chart will reveal the nature of the acid distilled.

Now it can readily be seen that mixtures of acids upon distillation must give curved instead of straight lines when depicted on the chart. With neighboring acids these curved lines will be very flat while the further removed from each other the acids are in their relative positions on the chart, the greater will be the bulge in the curve. Four examples are given to show the application of this principle to the actual analysis of mixtures of two acids.

Example 1.

Mixture of Formic and Acetic Acids.

0.5 cc. of formic acid required	128.9 cc. of 0.1 N KOH	($\frac{1}{2}$ =	64.45 cc.)
0.5 " " acetic " "	84.1 " " 0.1 " "	($\frac{1}{2}$ =	42.05 ")
Total	213.0 " " 0.1 " "	($\frac{1}{2}$ =	106.50 ")

The potassium salt solution of each acid was made up to a volume of 200 cc. in a graduated flask. A 100 cc. aliquot was taken from each flask, these two aliquots were mixed, and the 200 cc. volume so obtained was evaporated on the water bath to a volume of less than 30 cc. The residue was washed into a 100 cc. graduate, care being taken that the total volume in the graduate did not exceed 40 cc. From a burette was added enough 0.1 N sulfuric acid to bring the volume in the graduate to 100 cc. This was poured into the distilling flask, and then sufficient 0.1 N sulfuric acid was delivered into the graduate to make the total amount of acid used about 108 cc. (a slight excess to make sure that all the fatty acid would be liberated). A little water now added to the graduate brought the volume to 50 cc., and this was added to the 100 cc. already in the distilling flask. The acid so liberated and in solution in a volume of 150 cc. was distilled and the distillate collected in 10 cc. fractions for the first 100 cc. of the distillate, the second 100 cc. fraction of distillate being collected in a 100 cc. graduated flask. The data obtained are shown in Table VII.

TABLE VII.

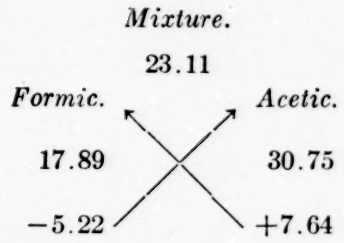
Mixture of Formic and Acetic Acids.

Collected, cc.....	10	10	10	10	10	10	10	10	10	10	100
0.1 N KOH, cc...	2.63	2.71	2.64	2.59	2.53	2.50	2.38	2.30	2.18	2.15	17.50
Acid,* per cent..	2.47	2.54	2.48	2.43	2.38	2.35	2.23	2.16	2.05	2.02	16.43
By addition, collected, cc.....	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**	**200**
Acid, per cent..	2.47	5.01	7.49	9.92	12.30	14.65	16.88	19.04	21.09	23.11	39.54

* Figures obtained by dividing the number of cc. of 0.1 N KOH required for each 10 cc. fraction by the total titration, *i.e.*, 106.5, and multiplying by 100.

Problem.—Given a mixture of two acids one of which, formic, has a distilling constant of 17.89, and another, acetic, which has a distilling constant of 30.75 (in both cases for the first 100 cc. fraction of distillate) in what proportion must they be mixed so that the mixture will have a distilling constant of 23.11 (for the first 100 cc. fraction of the distillate).

As already stated this calculation is one of the easiest, is known in every laboratory, and may be conveniently expressed on paper as follows:



Therefore, of 12.86 parts in all (corresponding to 106.5 cc. of 0.1 N KOH), 5.22 parts will represent the acetic acid and 7.64 parts will represent the formic acid.

$\frac{106.5}{12.86} = 8.2815$ cc. of 0.1 N KOH for each part.

$8.2815 \times 5.22 = 43.23$ cc. of 0.1 N KOH for the acetic acid.
$8.2815 \times 7.64 = 63.27$ " " 0.1 " " " " formic "

$0.0060042 \times 43.23 = 0.2596$ gm. of acetic acid determined.
$0.0060042 \times 42.05 = 0.2525$ " " " " taken.

$0.0046021 \times 63.27 = 0.2912$ gm. of formic acid determined.
$0.0046021 \times 64.45 = 0.2966$ " " " " taken.

Example 2.

Mixture of Formic and Acetic Acids.

1 cc. of formic acid required 215.11 cc. of 0.1 N KOH.
1 " " acetic " " 171.52 " " 0.1 " "
Total 386.63 " " 0.1 " "

This determination was intentionally made in a very rough manner with samples of acids different from those used in Example 1. 1 cc. of the formic acid was titrated with the 0.1 N KOH and the number of cc. required was noted as above. The titrated portion was then rejected. The same sample of acetic acid was used here as that indicated in Table III. 1 cc. of each of the acids was then added to 150 cc. of water and the distillation was carried out. The conditions of this distillation vary considerably from those in the previous case. Four times the amount of acid was distilled and, using the titration for total acid as indicated above, the acidity of the mixture distilled could be considered as only approximately correct. The distillate was collected as before; *i.e.*, in 10 cc. fractions (Table VIII).

TABLE VIII.

Mixture of Formic and Acetic Acids.

Collected, cc.....	10	10	10	10	10	10	10	10	10	10
0.1 N KOH, cc.....	10.33	10.17	10.10	9.80	9.45	9.42	9.13	8.88	8.62	8.32
Acid, per cent....	2.67	2.63	2.61	2.53	2.44	2.44	2.36	2.30	2.23	2.15
By addition, collected, cc.....	10	20	30	40	50	60	70	80	90	100
Acid, per cent....	2.67	5.30	7.91	10.44	12.88	15.32	17.68	19.98	22.21	24.36

Mixture.

24.36

Formic. *Acetic.*

17.89 30.75

−6.47 +6.39

Therefore, of 12.86 parts in all (corresponding to 386.63 cc. of 0.1 N KOH), 6.47 parts will represent the acetic acid and 6.39 parts will represent the formic.

$\frac{386.63}{12.86}$ = 30.07 cc. of 0.1 N KOH for each part.

30.07 × 6.47 = 194.55 cc. of 0.1 N KOH for the acetic acid.
30.07 × 6.39 = 192.15 " " 0.1 " " " " formic "

0.0060042 × 194.55 = 1.1684 gm. of acetic acid determined.
0.0060042 × 171.52 = 1.0298 " " " " taken (approximately).

0.0046021 × 192.15 = 0.8843 gm. of formic acid determined.
0.0046021 × 215.11 = 0.9900 " " " " taken (approximately).

Example 3.

Mixture of Acetic and n-Butyric Acids.

0.5 cc. of acetic acid required 84.8 cc. of 0.1 N KOH ($\frac{1}{2}$ = 42.40 cc.)
0.5 " " *n*-butyric " " 53.08 " " 0.1 " " ($\frac{1}{2}$ = 26.54 ")
Total 137.88 " " 0.1 " " ($\frac{1}{2}$ = 68.94 ")

After evaporating the combined potassium salt solutions of the acids to a volume somewhat less than 100 cc., the residue was made up to a volume of 100 cc. in a graduated flask and a 50 cc. aliquot was taken. This aliquot was made up to a volume of 150 cc., using a graduate, with the

requisite amount of 0.1 N sulfuric acid and water, and then distilled. The 10 cc. fractions collected gave the titrations shown in Table IX.

TABLE IX.

Mixture of Acetic and n-Butyric Acids.

Collected, cc.....	10	10	10	10	10	10	10	10	10	10	100	100	100	100
0.1 N KOH, cc..	4.68	4.30	3.92	3.63	3.31	2.96	2.62	2.28	2.00	1.89	14.80	7.84	4.91	2.97
Acid, per cent.	6.79	6.24	5.69	5.27	4.80	4.30	3.80	3.31	2.90	2.74	21.47	11.37	7.12	4.31
By addition, collected, cc.....	10	20	30	40	50	60	70	80	90	100	200	300	400	500
Acid, per cent.	6.79	13.03	18.72	23.99	28.79	33.09	36.89	40.20	43.10	45.84	67.31	78.68	85.80	90.11

Mixture.

45.84

Acetic. *n-Butyric.*

30.75 69.88

−15.09 +24.04

Of 39.13 parts in all (corresponding to 68.94 cc. of 0.1 N KOH), 24.04 parts will represent the acetic acid and 15.09 parts will represent the *n*-butyric.

$\frac{68.94}{39.13}$ = 1.762 cc. of 0.1 N KOH for each part.

1.762 × 24.04 = 42.36 cc. of 0.1 N KOH for the acetic acid.
1.762 × 15.09 = 26.59 " " 0.1 " " " " *n*-butyric acid.

0.0060042 × 42.36 = 0.2543 gm. of acetic acid determined.
0.0060042 × 42.40 = 0.2546 " " " " taken.

0.0088084 × 26.59 = 0.2342 gm. of *n*-butyric acid determined.
0.0088084 × 26.54 = 0.2338 " " " " taken.

Example 4.

Mixture of Formic and Isovaleric Acids.

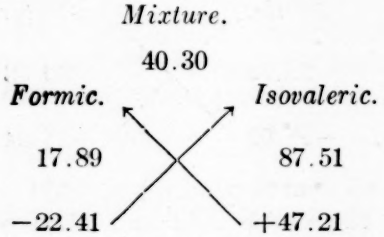
0.3 cc. of formic acid required 74.95 cc. of 0.1 N KOH ($\frac{1}{2}$ = 37.48 cc.)
0.4 " " isovaleric " " 34.73 " " 0.1 " " ($\frac{1}{2}$ = 17.37 ")
Total 109.68 " " 0.1 " " ($\frac{1}{2}$ = 54.85 ")

The potassium salt solutions of both acids were combined, evaporated to a low bulk, made up to 100 cc. in a graduated flask, a 50 cc. aliquot was taken, about 56 cc. of 0.1 N sulfuric acid were added, and enough water to make the total volume 150 cc. The mixture was then distilled. The results obtained are shown in Table X.

TABLE X.

Mixture of Formic and Isovaleric Acids.

Collected, cc.....	10	10	10	10	10	10	10	10	10	10	100	100	100	100
0.1 N KOH, cc..	4.11	3.36	2.86	2.47	2.10	1.87	1.58	1.39	1.26	1.10	6.98	4.24	3.08	2.36
Acid, per cent.	7.49	6.13	5.22	4.50	3.83	3.41	2.88	2.53	2.30	2.01	12.73	7.73	5.62	4.30
By addition, collected, cc.....	10	20	30	40	50	60	70	80	90	100	200	300	400	500
Acid, per cent.	7.49	13.62	18.84	23.34	27.17	30.58	33.46	35.99	38.29	40.30	53.03	60.76	66.38	70.68



The mixture consists of 47.21 titration parts of formic acid and 22.41 titration parts of isovaleric acid.

$\frac{54.85}{69.62} = 0.7878$ cc. of 0.1 N KOH for each part.

$0.7878 \times 47.21 = 37.19$ cc. of 0.1 N KOH for the formic acid.
$0.7878 \times 22.41 = 17.65$ " " 0.1 " " " " isovaleric acid.

$0.0046021 \times 37.19 = 0.1712$ gm. of formic acid determined.
$0.0046021 \times 37.48 = 0.1725$ " " " " taken.

$0.0102105 \times 17.65 = 0.1802$ gm. of isovaleric acid determined.
$0.0102105 \times 17.37 = 0.1774$ " " " " taken.

The curves illustrating these four examples have been drawn on the logarithmic chart. In the curve drawn for Example 4, for instance, it will be seen that the line crosses out acetic acid as a possible component of the mixture and then reaches a point on the chart where it begins to run parallel with the line which indicates formic acid. This identifies

absolutely the lower acid of the mixture and also gives some indication of the nature of the other acid. It will be seen in the case of Example 4 that the point reached where the line begins to run parallel is about 500 cc., showing that the other acid has run out of the mixture. Turning to Table V and reading across the table from the 500 cc. figure it will be found that the acid due to run out at this point is isovaleric. This is a little uncertain, however, and the qualitative tests given on page 467 are made use of to ascertain the nature of the higher acid of the mixture.

The Steam Distillation.

It might at first appear difficult, if not impossible, to so arrange the steam distillation that the volume in the distilling flask would be maintained at 150 cc. throughout the course of the distillation. In actual practice, however, this proved to be a very simple matter.

For the generation of the necessary steam the regulation steam-can heated by a gas burner was not tried because it seemed probable that such an arrangement would not suffice to maintain a steady and uninterrupted flow of steam to the distilling flask. The apparatus used for the purpose is one suggested by Lassar-Cohn:[4]

"The steam . . . . . is generated in a glass flask of 1 or 2 liters' capacity. This is half filled with water and, after the addition of a few drops of sulfuric acid, several pieces of zinc are added. During the boiling there occurs a gentle evolution of hydrogen and a regular and continuous passage of steam is thus obtained unaccompanied with bumping and tossing of the boiling water. If the suggested addition of sulfuric acid and zinc be neglected the water boils with much tossing and a very irregular passage of steam will result."

Technique of the Method.

The current to both hot plates is turned on and also the water leading to the condenser. Flask A is disconnected from the apparatus and into it are slid eight or ten pieces of stick zinc each about 1 inch in length. The flask is filled with ordinary water as hot as it can be procured to about the mark indicated in Fig. 2, and it is then tilted to get a fairly uniform distribution of the pieces of zinc over the bottom of the flask. The flask is

[4] Lassar-Cohn, Arbeitsmethoden für organisch-chemische Laboratorien, Hamburg, 1903, 3rd edition, p. 36.

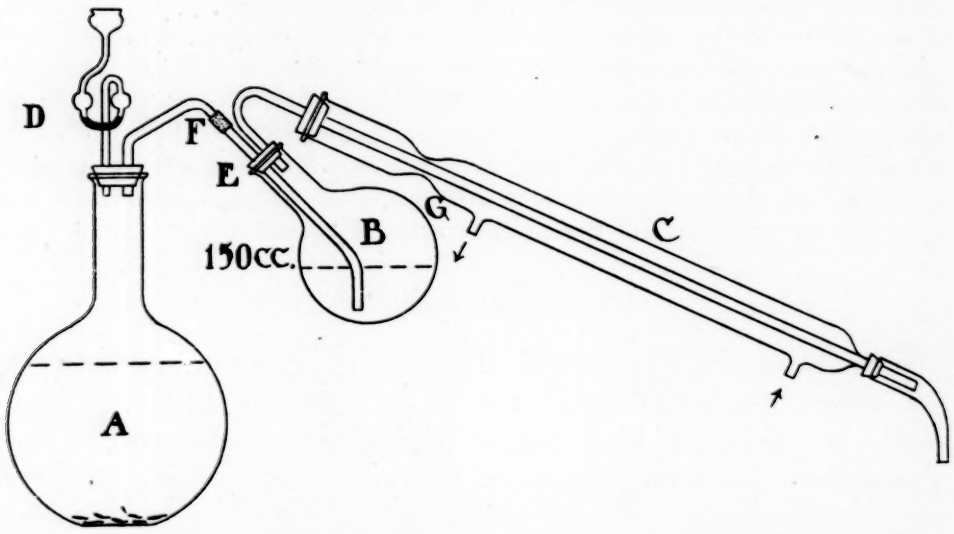


FIG. 2. Apparatus for use in the steam distillation. A, the flat-bottomed flask used for generating the steam, is of 2 liters' capacity. B, the distilling flask in which the volume of liquid is kept constantly at 150 cc. throughout the course of the distillation, is a round-bottomed one of 500 cc. capacity. Ordinary well fitting corks are used in the necks of these two flasks, and to connect the distilling flask with C, an ordinary Liebig condenser. D is a mercury seal made by pouring a little mercury into the thistle tube (there is very little pressure on this seal during the distillation). The glass tubes leading to and from Flask B are of as large a bore as the cork E will conveniently take (the ones used in this laboratory measured $\frac{5}{16}$ inch, inside diameter). F is a piece of rubber tubing to connect A with B. The tube leading from B reaches to a little below the neck of the condenser (at G).

Flask A is heated by an electric hot plate having a heating surface 6 inches in diameter, and during the distillation this plate is kept with the current at "full." Flask B is heated with a similar hot plate $4\frac{1}{2}$ inches in diameter. This plate is kept at "full" until the distillation is well started on its way, when it is turned to the next lowest step, "medium." This second hot plate may be placed on an iron ring which is clamped rather loosely to a stand so that it will swivel back out of the way when it is desired to remove Flask B. The apparatus is set up in a corner of the laboratory free from strong draughts and, when the distillation is once started, will run along for hours with little or no change in the 150 cc. volume. When the distillate is collected in 100 cc. fractions the usual work of the laboratory may be carried on without interruption. It is suggested that a trial distillation be made with 0.5 cc. of glacial acetic acid in 150 cc. of distilled water for practice in the use of the method and to determine the correct adjustment of the hot plates.

now placed on the larger hot plate, about 5 cc. of sulfuric acid (1:5) are added, the connecting cork is inserted in the neck of the flask which is then allowed to stand until the water boils vigorously but quietly.

The mixture of the acids in solution is titrated with the 0.1 N alkali solution and a note is made of the number of cc. required. It is advisable to have dissolved in the 150 cc. volume to be distilled an amount of acid equivalent to about 0.5 cc. of the pure acid.[5] A titration figure of 75 cc. of 0.1 N alkali will about represent an average amount in the case of the lower acids of the series, and 60 cc. if there is reason to believe that the mixture consists of the higher ones. It is now possible to figure the amount of dilution or concentration necessary so that the volume of the aliquot taken plus the volume of 0.1 or 0.2 N sulfuric acid required to liberate the fatty acids plus the requisite amount of water will make the total volume 150 cc. Here it may be well to observe that while a slight excess of acid over and above the amount necessary to liberate the fatty acids does no harm, a large excess will increase the rate of distillation of these acids to such a degree that the accuracy of the determination will be appreciably affected. The 150 cc. volume for distillation is made up in an ordinary 100 cc. graduate, in the manner described in the examples of distillations, and then transferred to the distilling flask.

As soon as the steam is seen issuing from the tube leading from the steam generating flask, A, the distilling flask, B, with its contents is attached to the apparatus, the smaller hot plate is swung into its supporting position under the flask, and a mark corresponding to the level of the liquid inside is made on the flask with a blue pencil. The distillation is then carried out. If oil drops or flakes collect in the condenser during the course of the distillation some one or more of the higher acids are present and the supply of cold water flowing through the condenser is turned off at once. The distillate is collected in two 10 cc. graduates using these alternately and reading to the lower meniscus for the 10 cc. mark. The first graduate, when filled to the mark, is removed and the second is substituted. The contents of the

[5] There is, however, considerable latitude in this, especially when the lower and more commonly occurring members of the series, which are more soluble, are distilled. (See the foregoing examples of distillations.)

first graduate are poured into a small Erlenmeyer flask and the graduate is washed out with three or four changes of distilled water. These washings are added to the contents of the flask. The graduate is then flipped vigorously to dislodge the drops of water from it, and set aside in an inverted position until required again. While waiting for the second graduate to fill the first fraction is titrated with the same 0.1 N alkali solution used for the total titration and the figure so obtained is divided by that representing the total titration. The result is multiplied by 100. The figure so obtained is the percentage amount of acid distilled into the first 10 cc. fraction of the distillate—the distilling constant.[6] It has been shown in the foregoing pages that the rate of distillation of a single acid is constant while, in the case of a mixture of acids, the rate of distillation is variable, depending upon the mixture of the acids.

After collecting the tenth 10 cc. fraction a 100 cc. graduated flask is placed under the condenser and the distillate from this point is collected in 100 cc. fractions. These are titrated in the same manner as the foregoing 10 cc. fractions, using phenolphthalein as indicator. The flasks are also washed out with distilled water, as in the case of the 10 cc. graduates, and used alternately. If fat particles adhere to the walls of the flasks they are cleaned out with bichromate mixture, washed thoroughly with distilled water, and then turned upside down to drain until needed for the succeeding fraction.

The number of cc. of 0.1 N alkali required for each succeeding fraction is divided by the total titration and the figure so obtained, after multiplying by 100, is added to the sum total of the figures preceding, which were obtained in the same manner (see examples of distillations). The figure representing the sum obtained with each successive addition is pointed off at its corresponding position on the logarithmic chart until the line of direction so indicated reaches a point from which it runs parallel with that of some one acid. This identifies with certainty the lower acid of the mixture and it is, of course, unnecessary to carry the distillation further. The point at which the line begins its parallel course is the exact place at which the other, and higher, acid of

[6] If the distillate is being collected in 100 cc. fractions this figure will be, of course, the distilling constant of the first 100 cc. fraction.

the mixture has run out. A reference to Table V will give an approximate idea as to which one it is. To establish definitely the nature of this acid, however, the included colorimetric tests must be used. A reference to the examples of distillations given in this paper will indicate the nature of the calculation necessary for determining the amounts of acids comprising the mixture.

The application of this method of steam distillation for the identification and determination of acids in solutions which cannot be titrated directly on account of their dark color, or for some other reason, is obvious. The solution is distilled directly from a total volume of 150 cc. and the distillates are collected either in 10 cc. or 100 cc. fractions. A calculation based on the following will illustrate the procedure.

Formula for Determination of the Total Amount of Acid Originally Present from the Titrations of the First and Second 100 Cc. Fractions of Distillate.

t = the total titration to be calculated (unknown).
t_1 = the titration of the first 100 cc. fraction (known).
t_2 = " " " " second 100 cc. " "
c = the distilling constant (unknown).

then:

$$c = \frac{t_1}{t},\ c = \frac{t_2}{t - t_1},\ \text{or}\ \frac{t_1}{t} = \frac{t_2}{t - t_1}.\quad t = \frac{t_1^2}{t_1 - t_2}$$

As an example (an unknown amount of acetic acid really requires 171.52 cc. of 0.1 N alkali):

First 100 cc. fraction of distillate (t_1) required 52.74 cc. of 0.1 N KOH.
Second 100 " " " " (t_2) " 36.52 " " " "
Difference 16.22

$$t = \frac{(52.74)^2}{16.22} = 171.49 \text{ cc. of 0.1 N KOH (representing the acidity of the acid solution distilled).}$$

$$c = \frac{t_1}{t} \text{ or } \frac{52.74}{171.49} = 30.75 \text{ (distilling constant).}$$

Conclusion: The acid originally present was acetic, and $0.0060042 \times 171.49 = 1.0297$ gm. of this acid present in the liquid distilled.

Confirmation: By the qualitative colorimetric method.

Colorimetric Tests.

These tests are based upon results obtained while working with a colorimetric test for the identification of the fatty acids

proposed by Agulhon.[7] The principle involved in this test of Agulhon's is good, but the method of carrying it out proved unsatisfactory.

A special study was made of the solubility of various metallic salts of the volatile fatty acids in various organic solvents. For this purpose the copper, iron, nickel, cobalt, chromium, manganese, and uranium salts of these acids were used. It was found that only the salts of iron and copper respond satisfactorily to tests of this nature. As a result of this investigation Agulhon's test has been rearranged, and the range has been somewhat extended through the addition of one or two other solvents. Agulhon states that his test may be nullified to some extent by the formation of insoluble copper salts with the use of copper sulfate, the reagent he indicates. This difficulty has been entirely removed by the substitution of copper chloride.

There is needed for this test a solution of iron chloride not weaker in strength than 2 per cent nor stronger than 3 per cent, and a solution of copper chloride of a strength approximating 0.1 N.

The 10 cc. fractions collected in the distillations are not rejected after the titration with the 0.1 N alkali but are made up to a certain volume so that the strength of the solution is approximately 1.5 per cent of the alkali salt of the fatty acid.[8] This volume may be obtained either by dilution, if too strong, or by evaporating off the excess of water, if too weak.

The test is made in the following manner. 2 cc. of the 1.5 per cent solution of the alkali salts of the fatty acids are trans-

[7] Agulhon, H., Solubilité de certains sels métalliques des acides gras volatils dans les solvants organiques,—Application à la détermination qualitative de ces acides, *Bull. Soc. chim.*, 1913, series 4, xiii, 404.

[8] Some judgment of the chemist is here called for. The first fractions of the distillate should be tested for the higher acids of the series and the last fractions for the lower acids. Unless there is some idea as to the nature of the acid, or acids, present in these fractions so that the corresponding factors may be used to obtain the approximate strength solution of the alkali salts of the fatty acids, the factor for propionic acid, which will about strike an average, may be used to multiply the number of cc. of 0.1 N alkali required to neutralize the fraction. When once prepared the alkali salt solutions of the volatile fatty acids should be tested within a reasonably short time since it has been found that molds may begin to grow in them after a few days

ferred by means of a pipette into a small test-tube (tubes 5 inches long and ½ inch in diameter were used in this laboratory) and from 0.5 to 1 cc. of the special solvent added, forming a supernatant layer. This mixture is then treated, drop by drop, with agitation, with either the iron chloride or copper chloride test solution, as indicated. The tube then remains at rest for a few moments and the result is noted.

Orientation Test.

To 2 cc. of the 1.5 per cent solution of the potassium or sodium salts of the fatty acids under investigation add about 0.5 or 1 cc. of amyl alcohol, stopper with the thumb, agitate the tube vigorously to mix the solvent and the solution, and then hold it under the hot water tap or plunge the tube into hot water, where it is held for several seconds with shaking, until the vapors of amyl alcohol have almost or entirely displaced the column of air from the tube. Cool, and then add one drop of the iron chloride solution. Stopper the mouth of the tube with the thumb, agitate vigorously, and set aside for a minute or two. If a brownish yellow precipitate is formed which hangs suspended in the aqueous liquid even for some minutes on standing, while the layer of amyl alcohol on the surface is colored a light yellow, the acid present may be either *heptylic*, *caprylic*, *pelargonic*, or *capric*. If the precipitate formed shows an inclination to dissolve readily in the solvent, add another drop of the iron chloride solution, stopper with the thumb, agitate vigorously, and again note the result. The iron salt of *propionic* acid is only partly soluble in the solvent. In this case the test will be indicated by a pronounced brownish yellow tinge of the amyl alcohol, by the collection of brown insoluble salts at the junction of the two liquids, while the aqueous solution will be colorless. With *acetic* acid the aqueous solution will be colored a clear urine shade of yellow containing the faintest tinge of red due to the dissolved iron salt of the acid itself, there will be no precipitate on standing, and the amyl alcohol layer will be colorless. With *formic* acid the amyl alcohol layer will be likewise colorless, but the aqueous solution will be cloudy and will give a red precipitate on standing for several minutes. If the amyl alcohol layer has begun to take

on a rich deep red after the addition of successive drops of the iron chloride solution, continue the addition of the drops with agitation until a full intense red has been developed. The iron salts of the three acids, *butyric, valeric,* and *caproic* are entirely soluble in the amyl alcohol and give the intense red color.

Individual Tests.

Formic and Acetic Acids.—No individual colorimetric test for formic acid could be found. It is necessary to make use of its reducing properties to identify it (the formation of a blue color with nitric acid and bichromate of potash mixture).[9] However, this acid may be identified with certainty in the manner noted under "Orientation Test," and in the steam distillation. Of all the solvents turpentine is the only one found which is able to dissolve the copper salts of formic and acetic acids and bring them to the surface of the aqueous solution.

In the absence of formic and the other acids, acetic acid may easily be identified by the following test. 2 cc. of the alkali salt solution of the fatty acid are placed in a small test-tube and covered with a 0.5 or 1 cc. layer of ordinary turpentine.[10] The approximately 0.1 N copper chloride solution is then added drop by drop from a burette, the mouth of the tube is closed with the thumb, and it is agitated after each addition. The copper salt of acetic acid will be dissolved by the turpentine and brought

[9] Agulhon, Recherche colorimétrique de l'alcool en présence de l'acétone. Réactions colorées de certains groupements organiques en présence d'acides minéraux et de bichromate de potassium, *Bull. Soc. chim.*, 1911, series 4, ix, 881.

[10] The solvent properties of the turpentine may be considerably increased by dissolving in it all the crude resin (rosin, colophonium) it will take up, which is very little. This may be done by triturating the turpentine with the powdered resin in an evaporating dish and decanting off the liquid. Redistilled turpentine cannot be used for this test. This turpentine test may also be used to distinguish between a volatile fatty acid and lactic acid; copper lactate is insoluble in the turpentine which rises as a colorless layer to the surface of the aqueous solution after agitation. It may also be noted here that lactic acid is practically non-volatile by the method of steam distillation described in this paper. The first 100 cc. fraction from 2 gm. of lactic acid dissolved in 150 cc. of water and steam-distilled required but 0.25 cc. of 0.1 N KOH.

to the surface of the solution as a deep greenish blue layer, provided the agitation has not been so great as to form an emulsion.

Propionic Acid.—Use the test given under "Orientation Test." The test with amyl alcohol and iron chloride was the only satisfactory one found. At first, add but one drop of the iron chloride solution. This will usually suffice to develop the brownish yellow tinge of the amyl alcohol. At the most, add but a few additional drops. If more than this is added the tendency of the insoluble salts formed is to drag the brown color out of the amyl alcohol, leaving it colorless.

Butyric and Valeric Acids.—Make the test as already indicated but use ether as the solvent[11] and iron chloride as the precipitant. If the acid present is butyric the two liquids will emulsify somewhat upon agitation, while the ether layer which eventually rises will be colorless. If the acid is valeric the ether layer will be reddish yellow in color while the aqueous solution will be decolorized. In both cases there will be a collection of insoluble salts at the junction of the two liquids.

Valeric, Caproic, and Heptylic Acids.—The first two acids mentioned are the only acids of the series which give color reactions with iron chloride and ether. Make the test as above described and set aside for a minute or two. The iron salts of valeric and caproic acids will color the ether layer a reddish yellow. In the case of the valerate the aqueous solution will be decolorized but very cloudy with insoluble iron salts if the acid present is caproic. In both cases there will be a collection of insoluble salts at the junction of the two liquids. The iron salt of heptylic acid is entirely insoluble in both the ether layer and the aqueous solution and there is no collection of insoluble salts at the junction of the two liquids.

Valeric and Caproic Acids.—Perform the test as usual, but with copper chloride and either petrol-ether (40–60°), gasoline, or kerosene, as the solvent. The copper salt of valeric acid is nearly all soluble in the aqueous solution, while the solvent layer rises colorless to the top. With caproic acid the solvent takes on a decided blue color.

Caproic and Caprylic Acids.—Perform the test as above de-

[11] It is preferable to use "ether over sodium" for these tests.

scribed, using the solution of iron chloride as precipitant and ethyl acetate free from alcohol as the solvent. If the acid present is caproic the aqueous solution will be decolorized while the solvent will possess a rich iron-red color. The iron salt of caprylic acid is almost entirely insoluble in both the solvent and the aqueous solution. At most, the solvent will possess but a faint yellow color while the aqueous solution will be very cloudy with insoluble salts.

SUMMARY.

A new and simple method of steam distillation for the determination of the volatile fatty acids has been devised. This method depends upon the maintenance of the aqueous solution distilled and which contains the acid, or acids, at a constant volume of 150 cc. throughout the course of the distillation. A simple and convenient apparatus for maintaining this volume is described.

By this method of steam distillation the distilling constants of the individual volatile fatty acids are indicated by straight lines when depicted on a simple logarithmic chart. On the other hand, the distilling variables of mixtures of these acids are indicated on the same chart by curved lines. With neighboring acids these curves will be very flat, while the further the acids comprising the mixture are removed from each other in their relative positions in the series the greater will be the bulge in the curve.

The lowest acid of the series in the mixture will be identified with certainty since it will eventually reach a point where it will begin to parallel some straight line on the chart which represents that acid. This same point indicates where the other acid of the mixture has run out and reference to a table given will indicate with a fair degree of probability the nature of this accompanying acid.

The calculations given which are necessary for the determination of the amounts of acid comprising the mixture are of the simplest, thus avoiding all errors. The application of these calculations to the determination of acids present in dark colored acid solutions and in bacteriological media where the acid present cannot be titrated directly has been shown.

A qualitative colorimetric test for the identification of these acids has also been included, and this may be used alone or in conjunction with the steam distillation method.

THE MECHANISM OF CYTOLYSIS IN SEA URCHIN EGGS.

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Because of its relation to the theory of hemolysis on the one hand and to that of membrane formation in artificial parthenogenesis on the other, the mechanism of cytolysis is a matter of interest and importance to the general physiologist. The purpose of the investigations described below is to determine the character of the change which brings about cytolysis.

In attempting to account for hemolysis, Koeppe[1] advanced the view that the outflow of hemoglobin from the blood cell is the result of the destruction of the limiting membrane of the corpuscle, which he believed to be a lipoid substance. Consequently he interpreted hot water hemolysis as a melting of the lipoid sac of each corpuscle, and referred fat solvent action to the solution of the lipoid shell.

Loeb,[2] considering the question with reference to the sea urchin egg, formulated a theory of cytolysis and membrane formation (superficial cytolysis) which was an elaboration and extension of Koeppe's hypothesis. According to Loeb's view, the lipoids of the cytoplasm are liquefied by various cytolytic agents, and as a result water is absorbed by the cytoplasm. This leads first to membrane formation and, if continued, to complete cytolysis; *viz.*, swelling and clearing of the entire egg.

Von Knaffl,[3] working under Loeb's direction, showed that, as a result of cytolysis, substances giving tests for lecithin flowed

[1] Koeppe, H., *Arch. ges. Physiol.*, 1903, xcix, 50.

[2] Loeb, J. Über den chemischen Charakter des Befruchtungsvorgangs, in Roux, W., Vorträge u. Aufsätze über Entwcklngsmechn. Organ., Leipsic, 1908, pt. ii.

[3] Von Knaffl-Lenz, E., *Arch. ges. Physiol.*, 1908, cxxiii, 279.

out of the sea urchin eggs. His results on hot water cytolysis seemed to indicate a melting point, 39–42°C., and thus gave support to a simple liquefaction theory of cytolysis.

As a result of later work,[4] it became evident that there is not a melting point for sea urchin egg lipoids, but rather a liquefaction range, 34–42°C., and further that there is a definite temperature coefficient for the process; *viz.*, 3 for 2°C. or about 200 for an interval of 10°C. This corresponds to a value of about 100,000 for μ in Arrhenius' equation:

$$K_1 = K_0 e^{\frac{\mu}{2}\left(\frac{T_1 - T_0}{T_1 T_0}\right)}$$

where K_0 and K_1 are the reaction velocity constants at the absolute temperatures T_0 and T_1, respectively; e is the base of the natural logarithms; μ is a constant. The above value for μ indicates that hot water cytolysis belongs to the same type of reaction as the destruction of bacteria at high temperatures and hot water hemolysis.[5]

While von Knafft's evidence would indicate that cytolysis is fundamentally a physical process, *i.e.*, a melting or a solution, the magnitude of the temperature coefficient may mean that the process is a chemical one. That cytolysis may be due to increased fluidity of certain egg constituents and at the same time follow the monomolecular law is shown by the fact that Schroeder[6] found that heating a gelatin solution at 100°C. for different lengths of time, reduced the viscosity of the solution at a rate characteristic of a monomolecular reaction.[7] He interpreted the change as due to a hydrolysis of the gelatin and called the phenomenon a "saponification reaction." Furthermore, Dunstan and Mussell[8] in 1911 showed that the courses of a number of reactions, such as acetic anhydride + $H_2O \rightarrow$ acetic acid, benzoyl

[4] Moore, A. R., *Quart. J. Exp. Physiol.*, 1910, iii, 257.

[5] Arrhenius, S., Quantitative Laws in Biological Chemistry, London, 1915, 55.

[6] Schroeder, P. v., *Z. physik. Chem.*, 1903, xlv, 75.

[7] R S. Lillie uses Schroeder's data on gelation to support the contention that the increase of fluidity in cytolysis is due to physical change, *Biol. Bull.*, 1915, xxviii, 295.

[8] Dunstan, A. E., and Mussell, A. G., *J. Chem. Soc.*, 1911, xcix, 565.

chloride + H_2O→ benzoic acid, acetoacetic ester → equilibrium mixture, aniline + ammonium thiocyanate → phenyl thiocarbamide, were accompanied *pari passu* by regular and progressive changes in viscosity. They were able in these cases to determine the reaction velocity constant for each reaction by means of viscosity measurements, which showed the reactions to be of the first order.

We may regard it as a fact established, that cytolysis is due in the main to an increase in fluidity (decrease in viscosity) of certain cell constituents, probably lipoids. A measure of cytolysis therefore becomes a measure of the fluidity of the system. This increase in fluidity may be due either to a simple melting or to a chemical reaction. From the foregoing it is apparent that our task is to decide between these two possibilities. Stated in another way, our problem becomes: Is cytolysis a monomolecular reaction?

It has been shown[4] that the progress of the cytolysis reaction, *i.e.*, the increase in the percentage cytolyzed during a given time interval can be followed step by step, simply by removing a pipetteful of eggs from the hot sea water from time to time, putting them into a watch-glass of cold sea water, and afterwards determining the percentage cytolyzed by counting several hundred eggs in each watch-glass. Such measurements give a basis for deciding the character of the reaction, whether it is a physical process involving only the relation of molecules to each other, or to a chemical reaction involving a transformation within the molecule, such as a hydrolysis.

The fact that the percentage of eggs cytolyzed at a given temperature, $T°$, is a function of the time for which $T°$ is maintained may be interpreted in one of two ways:[9]

1. The time at $T°$ necessary for an egg to cytolyze may be due chiefly to the age of the egg, in which case the reaction should be slow at first, most rapid when the process is half completed, and fall off to zero at the end. If such were the case, the time plotted as abscissæ against $\log_e N$ (N, number of eggs remaining uncytolyzed at the end of the time interval taken) as ordinates, would give a double curve graph.

[9] Arrhenius, Quantitative Laws in Biological Chemistry, London, 1915, 78.

2. The increase in the number cytolyzed with time may amount to a certain percentage of the eggs remaining uninjured at the end of each time interval, and therefore be independent of the age or natural resistance of the egg. Such a dependence on the law of probability is shown by chemical reactions. In this case the graph resulting from plotting time as abscissæ against $\log_e N$ as ordinates must be a straight line.

Figs. 1, 2, 3, and 4 show that hot water cytolysis fulfills the requirements of the second case and is therefore of the nature of a chemical reaction.

The temperature coefficient of the process and the logarithmic relation of the percentage change to time both indicate hot water cytolysis to be of the nature of a chemical reaction. If this conclusion is correct, then it should be possible to determine the order of the reaction.

Using the method described, a large number of measurements of the percentages of cytolysis at various temperatures were made. It is obvious that on account of the variations between the lots of eggs from different individuals, and because we are forced to determine each percentage from a few hundred eggs, the experimental errors are large. Nevertheless, repeated tests made in different seasons, with the fresh unfertilized eggs of the sea urchin, *Strongylocentrotus purpuratus*, have given concordant results. In the table, each one of Experiments 1, 2, 3, and 4 represents results derived from the eggs of one individual sea urchin. Experiments 5 and 6 show the rates of cytolysis in two lots of eggs from the same female, tested at the same time; in Experiment 5 the eggs were normal unfertilized; in Experiment 6 the eggs were kept for 10 minutes in a solution of $SrCl_2$ 3/8 M before being put into the heated sea water.

In testing the hypothesis set forth above, whether cytolysis is due to a monomolecular reaction, the form of the equation used is

$$K = \frac{\log_e N_0 - \log_e N_1}{t_1 - t_0}$$

in which N_0 is the percentage of eggs uncytolyzed at the beginning of the experiment t_0; N_1 is the percentage of eggs uncytolyzed at the end of a given time t_1; K is the reaction velocity constant. In case the calculations were made from $t_0 = 0$ the first constants

TABLE I.

t	N	$\text{Log}_e N$	$K = \frac{\text{Log}_e N_0 - \text{Log}_e N_1}{t_1 - t_0}$
Experiment 1. $T = 35°$. $t_0 = 10$.			
10	98	4.58	
20	88	4.47	0.011
30	55	4.00	0.029
40	51	3.93	0.022
50	33	3.49	0.027
Experiment 2. $T = 36°$. $t_0 = 10$.			
10	97	4.57	
20	68	4.21	0.036
30	55	4.00	0.0285
40	40	3.68	0.030
60	33	3.49	0.022
Experiment 3. $T = 39°$. $t_0 = 2$.			
2	91	4.51	
4	44	3.78	0.36
6	33	3.49	0.25
8	12.5	2.52	0.33
10	10	2.30	0.28
Experiment 4. $T = 40°$. $t_0 = 4$.			
4	97	4.57	
5	90	4.49	0.080
7	75	4.31	0.086
9	65	4.17	0.080
11	60	4.09	0.069
13	50	3.91	0.073
Experiment 5. $T = 37.5°$. $t_0 = 3$.			
3	100	4.600	
5	99	4.589	0.005
10	95	4.549	0.007
20	85	4.439	0.0095
Average.....			0.0072
Experiment 6. $T = 37.5°$. $t_0 = 0$.			
3	85	4.44	0.053
5	65	4.17	0.086
10	35	3.55	0.105
20	20	2.99	0.080
Average.....			0.081

were invariably low in value. This may be due, as Arrhenius suggests,[10] to the necessity for an incubation period in the reaction.[11] The constants calculated from $t_0 = t$ of the first experimental reading show a fair degree of agreement. Hence we must conclude that the increase in fluidity which gives rise to cytolysis is due to a simple monomolecular reaction, possibly a hydrolysis. It will be seen that such a conclusion is in accord with Schroeder's theory and with the results of Dunstan's and Mussell's elaborate investigations.

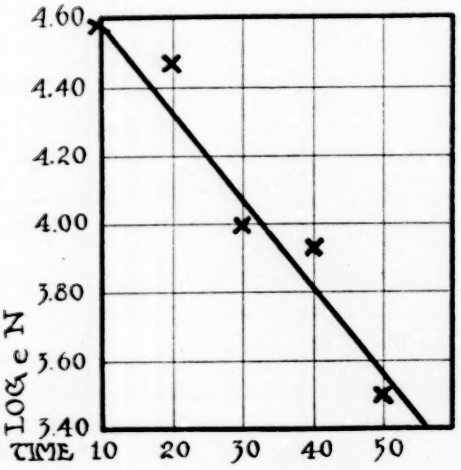

FIG. 1. From Experiment 1.

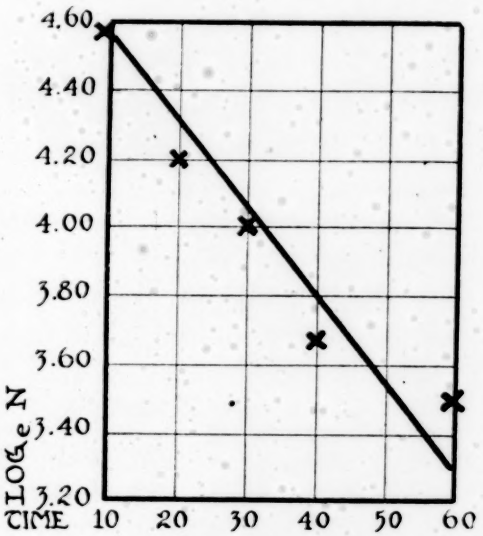

FIG. 2. From Experiment 2.

The Effect of Strontium Ions on the Reaction Velocity of Cytolysis.

Loeb[12] has shown that treatment of the unfertilized eggs of the sea urchin with $SrCl_2$ solution causes them to form fertilization membranes (superficial cytolysis) when treated with foreign serum. Robertson[13] has shown that $CaCl_2$ solution acts in the

[10] Arrhenius, Quantitative Laws in Biological Chemistry, London, 1915, 64.

[11] Charging the cylinder of heated sea water with the pipetteful of eggs necessarily reduces the temperature at the outset of the experiment by 0.2–0.4°C.; experimental temperature is recovered in 3 to 5 minutes.

[12] Loeb, Artificial Parthenogenesis and Fertilization, Chicago, 1913, 195.

[13] Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1912–13, xxxv, 88.

same way. The writer found[13] that the eggs of *Strongylocentrotus purpuratus* could be fertilized with starfish sperm if first sensitized with $SrCl_2$, and that the rate of hot water cytolysis increases enormously if the eggs have been kept for a few minutes in 3/8 M $SrCl_2$ solution before being put into the heated sea water. A consideration of Experiments 5 and 6 shows that the magnitude

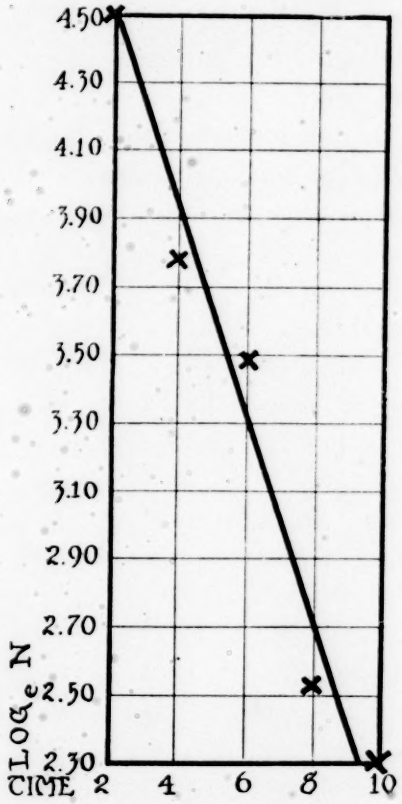

FIG. 3. From Experiment 3.

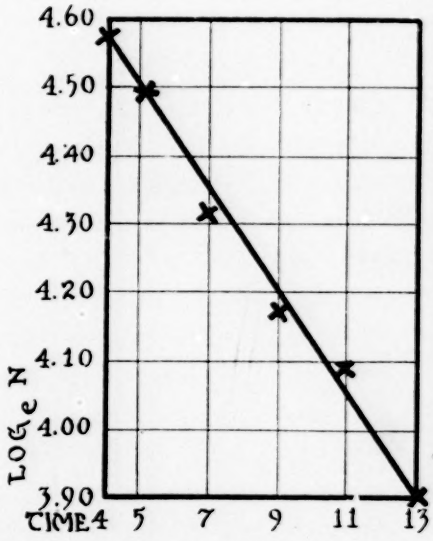

FIG. 4. From Experiment 4.

of the velocity constant of cytolysis at 37.5°C. for the eggs treated with $SrCl_2$ solution is eleven times that of the control. Since the $SrCl_2$ adds no energy to the system, it must therefore influence the reaction as a catalyzer. It is interesting and doubtless pertinent to note that Osterhout[15] regards the specific effect of the Ca ion on protoplasm as due to its action as a catalyzer of certain

[14] Moore, *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvii, 433.

[15] Osterhout, W. J. V., Marine Biological Laboratory Lectures, Woods Hole, 1916.

metabolic reactions. The suggestion may then be made that the sensitizing action of Sr and Ca ions on sea urchin eggs may be due to the fact that these ions act as catalyzers of the cytolysis reaction.

CONCLUSIONS.

1. Hot water cytolysis of the fresh unfertilized eggs of the sea urchin depends upon an increase in the fluidity of certain of the eggs' constituents, which change is caused by a process proceeding at the rate of a reaction of the first order.

2. Strontium ions act as a positive catalyst of the cytolysis reaction. The action of strontium ions in sensitizing sea urchin eggs to foreign sera and to starfish sperm may be due to catalysis of the cytolysis reaction.

IS LYSINE THE LIMITING AMINO-ACID IN THE PROTEINS OF WHEAT, MAIZE, OR OATS?*

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(From the Laboratory of Agricultural Chemistry of the Wisconsin Experiment Station, Madison.)

PLATE 4.

(Received for publication, November 27, 1916.)

Data published in 1914 by Osborne and Mendel[1] were interpreted by them as indicating that maintenance but not growth of an animal is possible without the amino-acid lysine. This created the impression in the minds of several investigators that this amino-acid is the limiting factor in determining the value of many proteins of vegetable origin.[2] The analytical data secured by Osborne, Van Slyke, Leavenworth, and Vinograd[3] on a number of plant proteins support this interpretation. They conclude that the lysine content of the proteins of the endosperm of wheat, maize, and rice is respectively 1.58, 0.97, and 4.26 per cent of the total nitrogen, and state that "It is rather striking that the figures for the utilizability of these proteins correspond so closely with the lysine which they yield."

Buckner, Nollau, and Kastle[2] interpreted the experimental data of Osborne and Mendel[1] as showing that "lysine is primarily responsible for the stimulation of growth." They fed to chicks such complex mixtures as wheat, wheat bran, sunflower seed, hemp seed, and skim milk, and contrasted the results with those obtained with a ration which consisted of a mixture of barley, rice, hominy, oats, and gluten flour. They interpreted

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 325, 334.

[2] Buckner, G. D., Nollau, E. H., and Kastle, J. H., *Am. J. Physiol.*, 1916, xxxix, 132 (and 21); *Kentucky Agric. Exp. Station, Bull. 197*, 1916.

[3] Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.

the results of these feeding experiments as making it evident that the marked differences shown by the two lots of chicks in rate of growth and development are to be ascribed to the "differences in the amino-acid content of the two rations and in all probability to differences in the lysine content."

The findings of Osborne[3] that gliadin purified as far as possible still yields 1.21 per cent of lysine naturally invalidate the earlier deductions of Osborne and Mendel that lysine is dispensable from the diet during maintenance.

In more recent work by Osborne and Mendel[4] they employed corn gluten, containing about 1 per cent of lysine, together with 1.33 per cent of N × 6.25 as "protein-free milk" as the sole source of protein. They compared the growth obtained with this protein mixture with that obtained with rations similar in their make-up except that the corn gluten and "protein-free milk" nitrogen was supplemented with lactalbumin and cottonseed flour respectively. The latter additions are known to yield the amino-acid lysine in relatively high amounts. The authors state that their results confirm the conclusions drawn by Buckner, Nollau, and Kastle respecting the effect of foods high and low in lysine on the growth of chicks.

In the course of our studies directed toward ascertaining the supplementary relationships among the naturally occurring foodstuffs, we have secured data which bear on this subject in a definite way. The results leave no room for doubt that a generalization cannot be safely made that any one amino-acid, as lysine, is present in the protein mixture in our more common vegetable foods in amount relatively smaller than are other essential amino-acids. Indeed we are forced to the conclusion that in the protein mixture of the maize kernel and the oat kernel, lysine certainly is not the essential protein cleavage product which is present in amount so small that it is the limiting factor which determines the biological value of the proteins of these seeds.

Our rations were made up after the following plan. A food mixture consisted of a single grain so supplemented with inorganic salts and butter fat as to be properly constituted for promoting growth and prolonged well-being, except that the protein was too low in amount to promote growth at the normal rate. In

[4] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxvi, 293, 299.

all cases our rations were shown to be made highly efficient for growth by the addition of purified protein alone, when casein was employed. Casein is well known to be a complete protein, and to suffice as the sole source of nitrogen throughout the life of the rat.[5]

Rations made up in this way were fed without the addition of supplementary protein and also with the addition of the incomplete proteins zein and gelatin respectively. The former yields no lysine while the latter yields about 6 per cent.[3] Obviously if lysine were the one amino-acid whose addition is necessary in order to improve the protein mixture of the grain employed in the food mixture there should be a response with growth when gelatin was added and no improvement when zein was added.

For completeness we also present curves of growth showing the effect of adding wheat gluten, the proteins of which yield but little lysine (1.58 per cent)[3] to rations which aside from the wheat gluten addition, derived their protein solely from the wheat, maize, and oat kernels respectively.

Our results may be briefly summarized as follows:

1. Zein does not supplement the protein mixture in the wheat kernel or the maize kernel so as to improve the rate of growth of young rats (Charts 3 and 10).

2. Zein does supplement the proteins of the oat kernel in a surprisingly efficient manner (Chart 7) although it lacks tryptophane and lysine and is one of the poorest of the proteins in cystine. In the proteins of the oat kernel, therefore, the above amino-acids are eliminated as being possibly the essential protein cleavage products present in minimal amount, and determining the plane of intake essential for growth.

[5] This statement rests on our experience reported elsewhere (*J. Biol. Chem.*, 1915, xxiii, 231) of observing growth at about the normal rate on a diet in which 99 per cent of the nitrogen was furnished by casein and 1 per cent by the alcoholic extract of wheat germ, added for the purpose of supplying the still unidentified dietary factor, the water-soluble B. 28 per cent of "protein-free milk," containing 0.76 per cent of nitrogen, which serves the same purpose as our extract, furnished, when fed with 18 per cent of purified proteins, 6.9 per cent of the total nitrogen of the food mixture in forms not further characterized except that we have found it not to be without biological value as a source of the cleavage products of protein (*J. Biol. Chem.*, 1915, xx, 415).

3. Gelatin chemically supplements the protein mixture of both the wheat kernel and oat kernel respectively. Since gelatin contains no tyrosine or tryptophane, and but a trace of cystine, but contains 6 per cent of lysine, it is evident that neither tyrosine, tryptophane, nor cystine is the limiting amino-acid in these grains. It tends to support the view, but does not prove that lysine is the amino-acid whose addition alone to the wheat protein mixture raises the biological value of the latter.

4. The addition of wheat gluten to either the wheat or maize kernel proteins supplements them so as to improve growth. The proteins of the wheat gluten are probably as a mixture qualitatively adequate as respects all of the indispensable amino-acids, although we cannot look upon this point as definitely established. One of its constituents, glutenine, has been shown by Osborne and Mendel to be capable of supporting growth when it furnished 93.2 per cent and "protein-free milk" 6.8 per cent.

It seems probable, therefore, that our combinations of wheat kernel proteins with wheat gluten, and of maize kernel proteins with wheat gluten led to growth because of a higher intake of protein rather than to a supplementary relationship between the proteins from the two sources in the sense of one making good the amino-acid deficiencies of the other. The results of feeding maize proteins with wheat gluten are of particular interest, however, because of their pronounced effect in promoting growth despite the relatively low lysine content of both the wheat and maize proteins.

Gelatin with its high lysine content does not improve the proteins of the maize kernel.

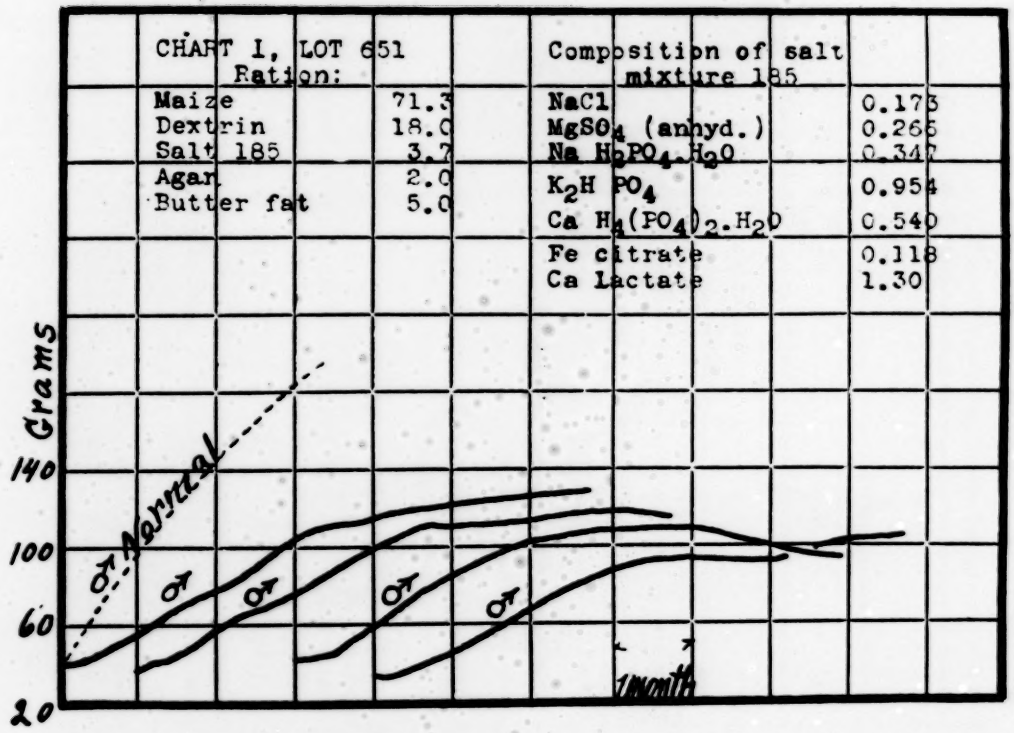

CHART 1. Lot 651 shows the growth curves of rats whose protein supply is limited to that contained in 71.3 per cent of ground maize. The salt mixture and butter fat included in the food mixture make satisfactory the inorganic content and the content of the fat-soluble A. The maize contains an abundance of the water-soluble B.[6] The one dietary factor which is responsible for the stunting and ultimate suspension of growth is the insufficient amount and poor quality of the protein in this food mixture. When 10 per cent of protein of wheat gluten is added to this diet it becomes adequate for normal growth (Chart 4). A similar improvement in growth should result in some degree from the addition of a single amino-acid, if we knew which of the essential ones is present in limiting amount in the mixture of maize proteins found in the seed. We have supplemented this ration with gelatin and with zein in order to ascertain whether lysine is the limiting amino-acid in the maize proteins (compare Charts 2 and 3).

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[6] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 153.

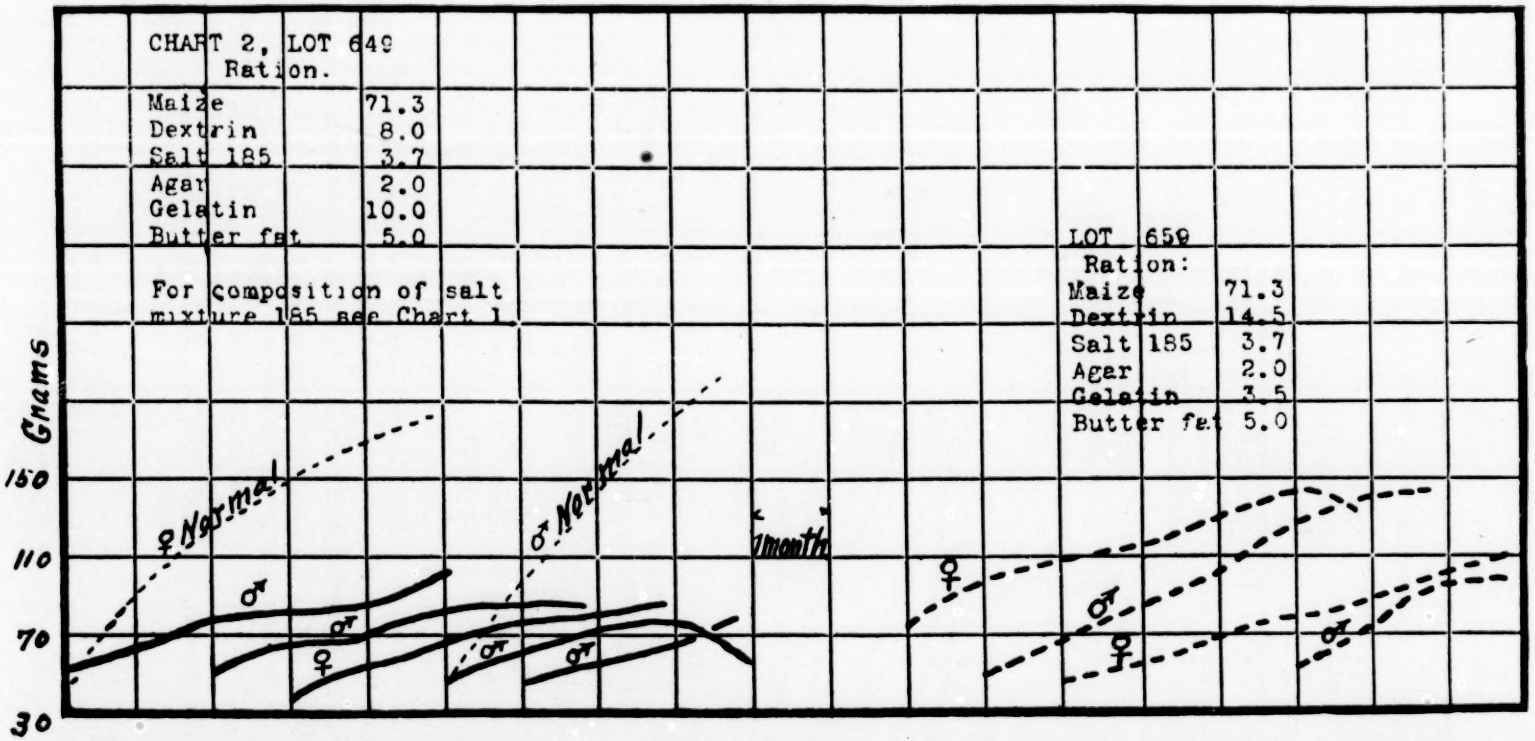

CHART 2. Lots 659 and 649 illustrate that gelatin, when added to the proteins of the maize kernel, does not supply the essential amino-acid which is present in smallest amount and therefore the limiting factor. Gelatin is exceptionally rich in lysine (6 per cent). The results of these feeding experiments indicate that lysine is not the limiting factor which determines the biological value of the maize proteins. 3.5 per cent of gelatin supplies the same amount of lysine as does 10 per cent of wheat gluten proteins,[7] yet the latter addition greatly improves our standard ration in respect to its protein content (compare Charts 1 and 4), while the former is without beneficial effect. The rats in this group not only suffered stunting but also nearly complete loss of hair (Fig. 1). There were, however, no signs of an eczematous condition of the skin, which we have frequently observed, especially on the nose, ears, and tail of animals suffering malnutrition from improperly constituted diets.

[7] The wheat gluten employed contained 76 per cent of protein.

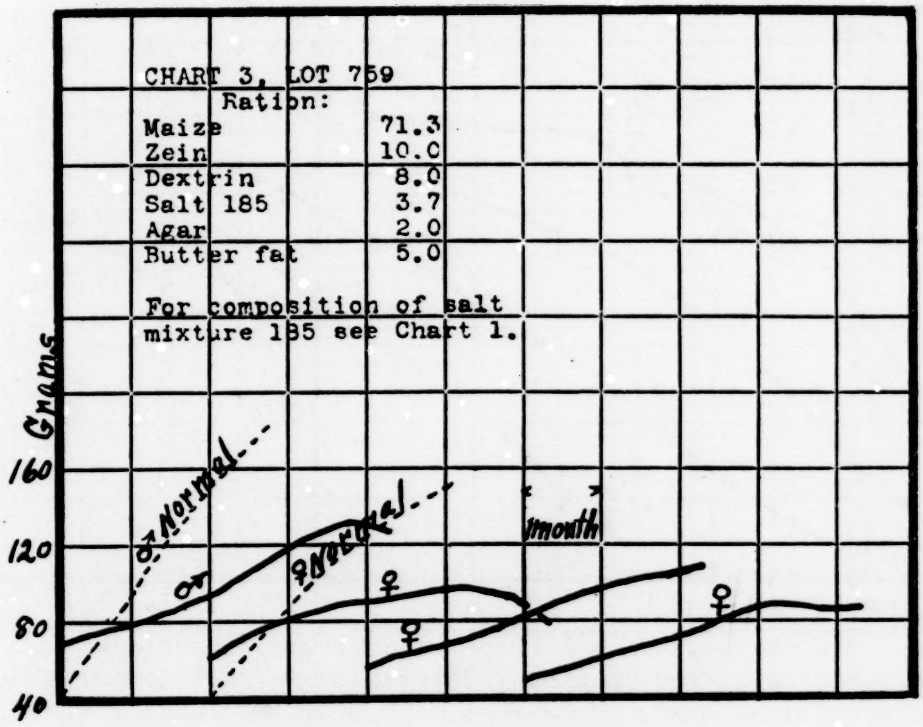

CHART 3. Lot 759 shows that the addition of zein to 7.13 per cent of maize proteins does not increase the biological value of the latter in a noticeable degree (compare Charts 1 and 3).

Zein contains no lysine. In marked contrast to its failure to supplement the proteins of the maize kernel is the pronounced benefit resulting from the addition of zein to a diet the protein content of which is inadequate in amount and which is wholly derived from the oat kernel (Chart 7).

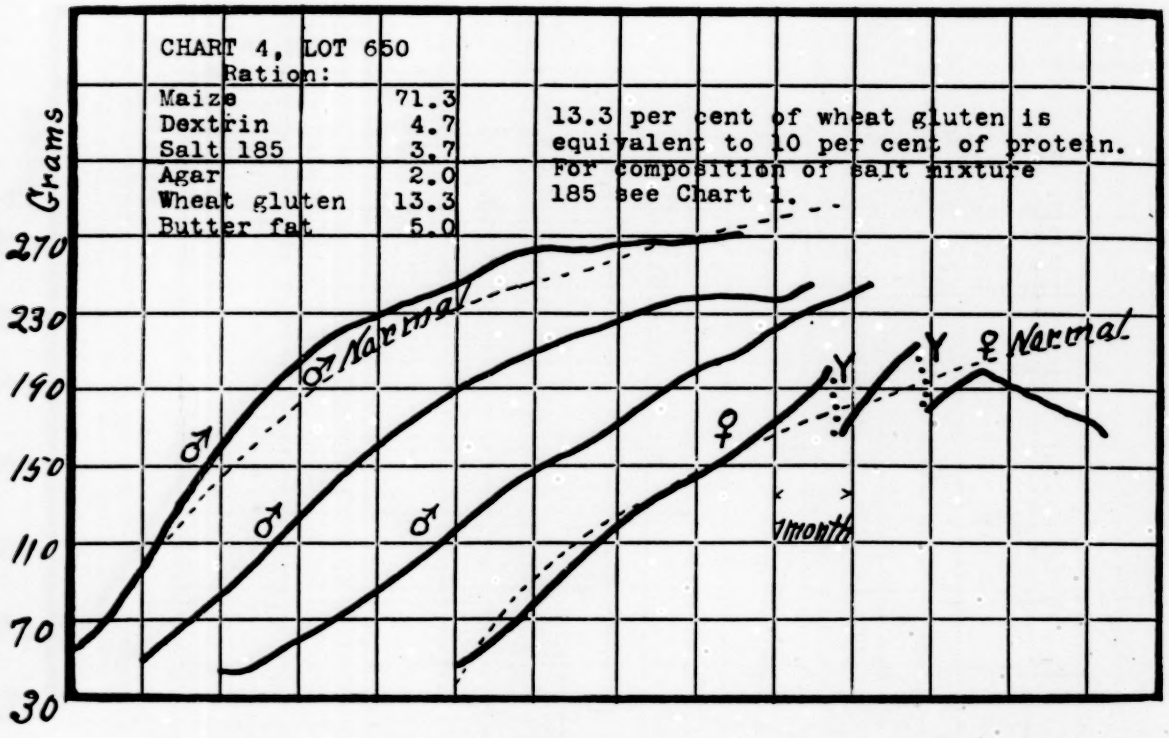

CHART 4. Lot 650 shows the great improvement in growth and well-being of rats fed the same ration as those described in Charts 1 to 3 inclusive but with 10 per cent of protein added in the form of wheat gluten. 10 gm. of the protein of wheat gluten yield the same amount of lysine as do 3.5 gm. of gelatin. Gelatin additions do not improve the maize proteins in the slightest degree. We interpret these results as indicating that lysine is not the limiting amino-acid which determines the biological value of the maize proteins. Since the protein mixture in either the maize or the wheat kernel is qualitatively adequate for growth, there is no evidence from the results here described that there is a true supplementing action between wheat and maize proteins. The good growth is due rather to the high content of a mixture of proteins of low value. The relative lysine contents of wheat gluten proteins and gelatin serve to emphasize, however, that lysine addition does not raise the value of maize protein for growth. In Fig. 2, the rat on the right shows the appearance of the animals fed on this mixture.

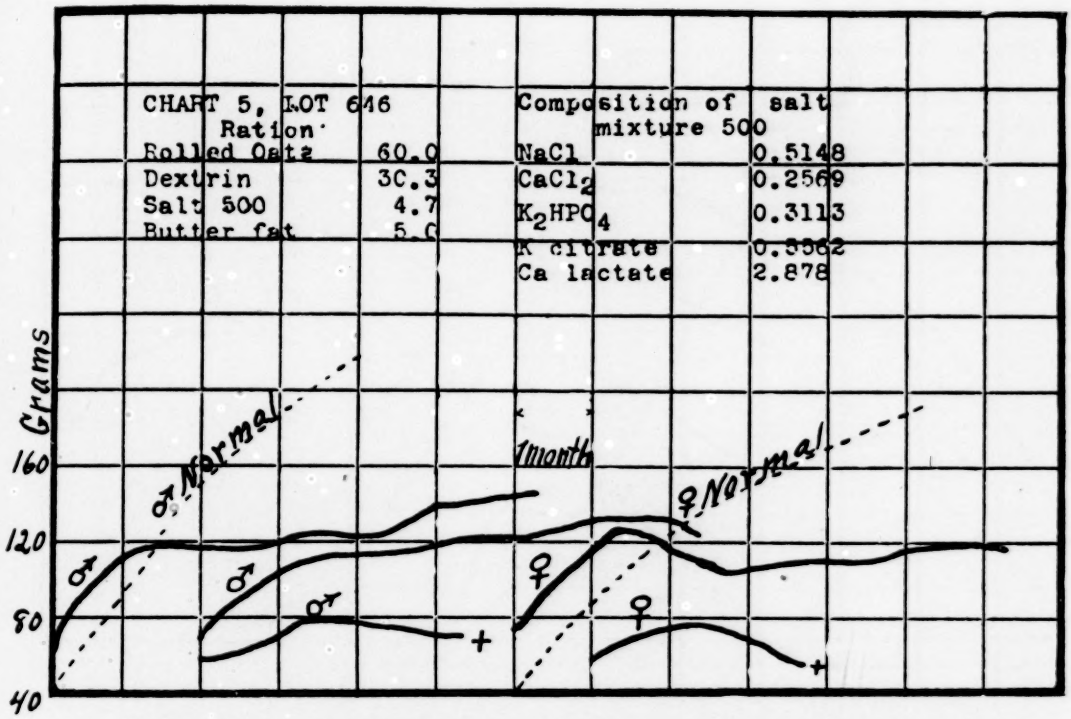

CHART 5. Lot 646 illustrates the character of the growth curves of rats fed a ration all the factors of which were properly adjusted except that the content of protein (9.0 per cent) which was derived solely from rolled oats was too low for normal nutrition. This food mixture, when a part of the dextrin is replaced by protein of suitable quality, becomes adequate for complete growth (Charts 6 and 7). We have selected this ration for the purpose of testing whether it is better supplemented by a protein yielding a high proportion of lysine (gelatin) than by one yielding low lysine (wheat proteins) or one yielding no lysine (zein). The results (Charts 6, 7, and 8) show that during the first 4 months the addition of zein led to greatly improved growth, despite the absence of lysine.

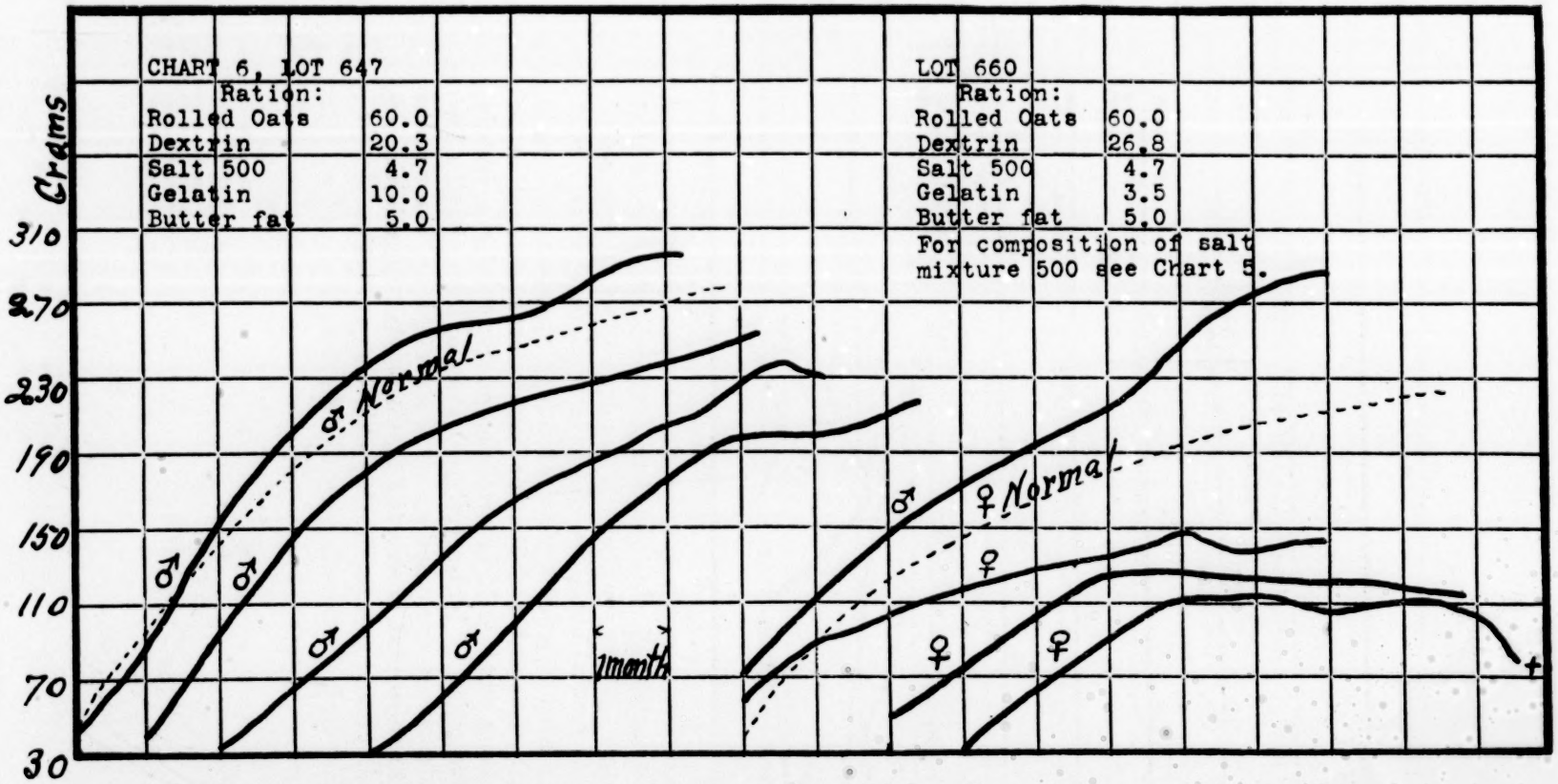

CHART 6. Lot 647 illustrates the fact that our standard mixture (Chart 5) with its 9 per cent of oat protein, becomes adequate to support growth at the normal rate to the full adult size when 10 per cent of gelatin is added. This serves as a remarkable illustration of the need of an understanding of the degree to which the proteins of one natural foodstuff make good the supply of the cleavage products of another which are present in smallest amount. Gelatin, as a source of protein, is wasted when combined with maize proteins, but its nitrogen is of value when combined with oat or wheat proteins (compare Charts 5 and 6 with 1 and 2, and with 9, 11, and 12).

Lot 660 shows that the oat proteins are improved by the addition of 3.5 per cent of gelatin (compare Charts 5 and 6). This improvement is hardly to be attributed to the lysine content of the gelatin, since the addition of zein, which contains no lysine, likewise enhances the value of the oat protein (Chart 7) doubtless by supplying an additional amount of the essential amino-acid present in smallest amount in the proteins of the oat.

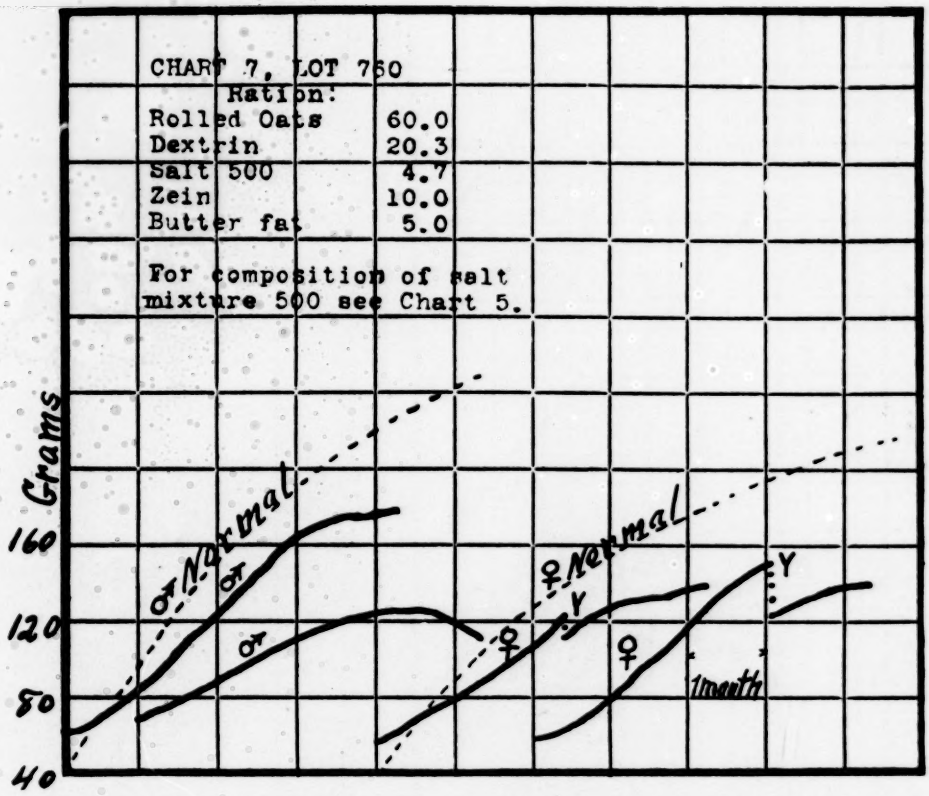

CHART 7. Lot 760 when compared with Lot 646 (Chart 5) shows the marked benefit to the animals which resulted from superimposing 10 per cent of zein upon the 9 per cent of oat protein contained in the basal ration employed in Charts 5 to 8 inclusive. On this ration with dextrin replacing the zein we have never seen rats grow beyond the 5th week. The addition of zein enabled them to grow at nearly the normal rate during 4 months and led to the production of two litters of young. The young were not reared. We interpret this result as leaving no doubt that a protein which does not yield lysine[8] serves to enhance the value of the proteins of the oat kernel, and seems to exclude the possibility of lysine being the limiting amino-acid in the protein mixture of the oat kernel.

[8] Osborne, T. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1913, xiv, 481.

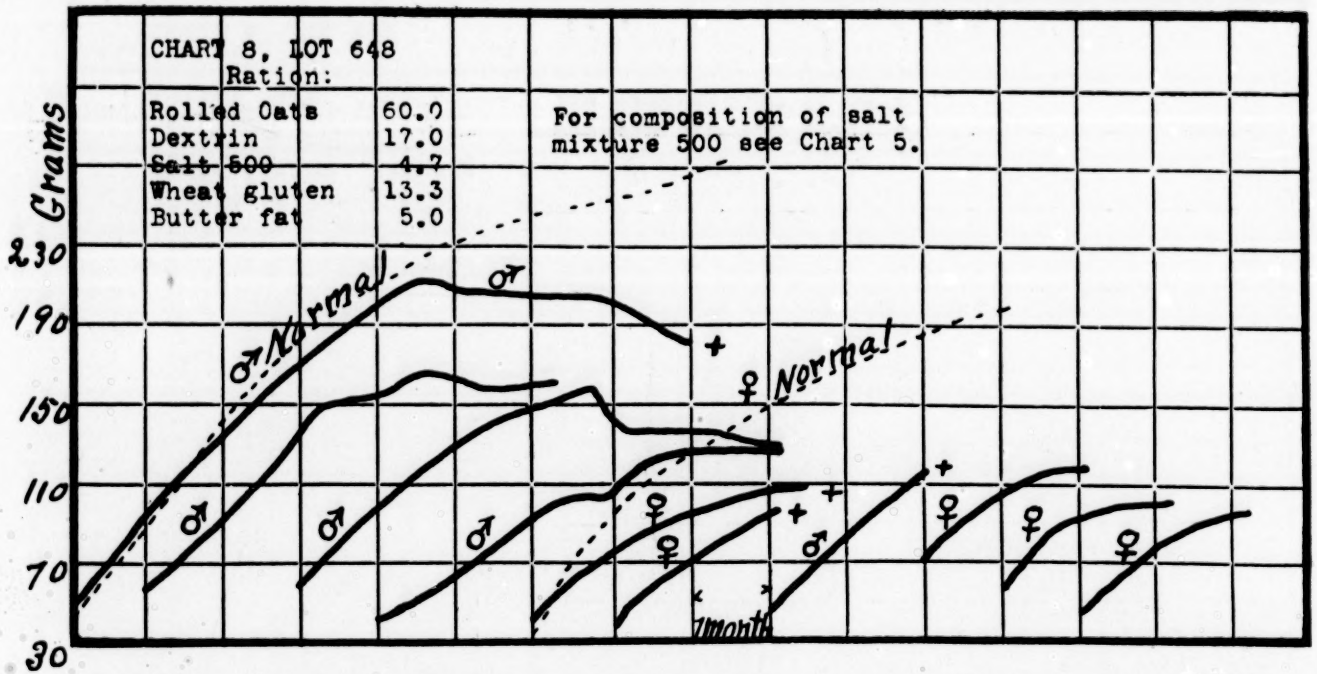

CHART 8. Lot 648 shows the result of superimposing 13.3 per cent of wheat gluten (equivalent to 10 per cent of protein) upon the oat proteins of our standard mixture (Chart 5). There is some improvement in the condition of the rats as a result of this addition as indicated by their ability to grow even to the 4th and 5th month, whereas without protein supplement the ration does not support growth beyond the 5th week (Chart 5). All the ten animals we have fed with the ration of Lot 648 have suffered suspension of growth at an early age, and the mortality in early life was high. We are inclined to believe that oats fed in liberal amount are injurious to rats. This we know to be true for wheat products[9] and it would seem to be plausible that in the present instance there may be in some degree a supplementary relationship between the wheat and oat proteins whereby the latter are improved in character. There is a demonstrable injury due to the combined injurious action of the wheat gluten and oats.[9]

[9] Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C., *Wisconsin Agric. Exp. Station, Bull. 17*, 1911.

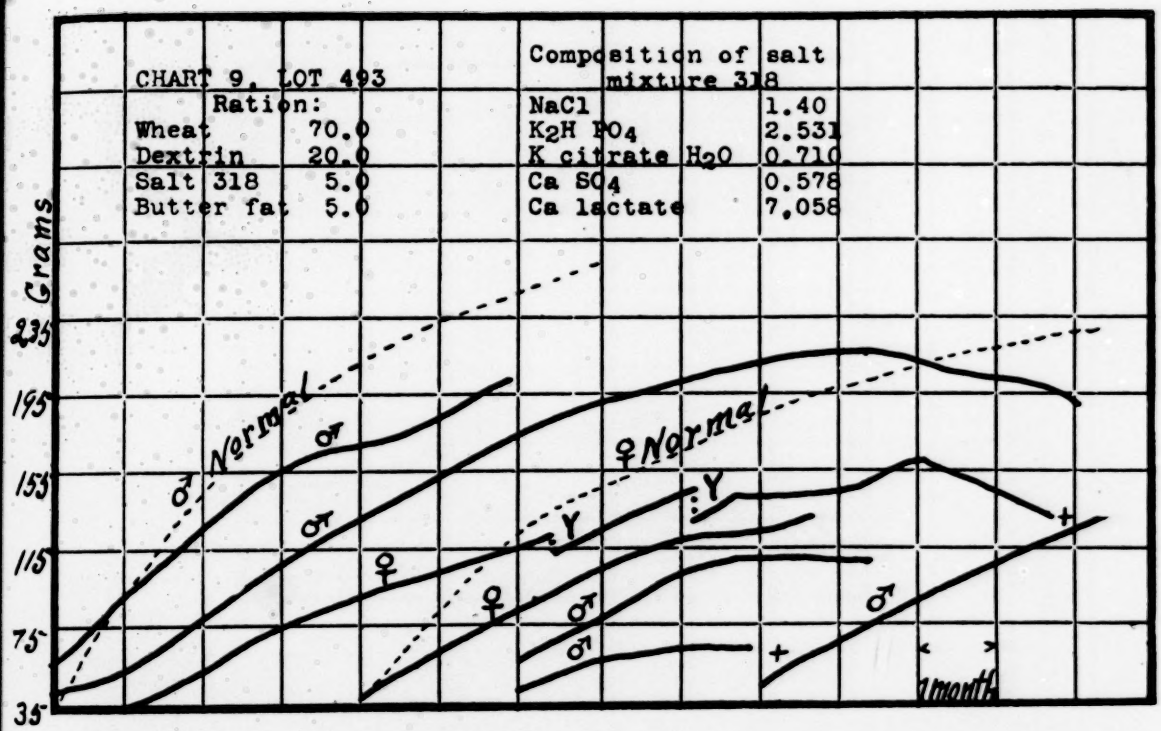

CHART 9. Lot 493 shows the degree of depression of growth in rats when the protein content of the diet is all derived from 70 per cent of wheat (7 per cent of protein). The substitution of a part of the dextrin of this food mixture by a suitable protein renders it adequate for the support of normal growth and reproduction.[10]

We have employed this mixture as a basal ration to which we have added (a) zein and (b) gelatin, to ascertain whether the supplementing action of these incomplete proteins would indicate whether lysine is the limiting amino-acid in the protein mixture in the wheat kernel (compare Charts 10, 11, and 12).

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[10] McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 352.

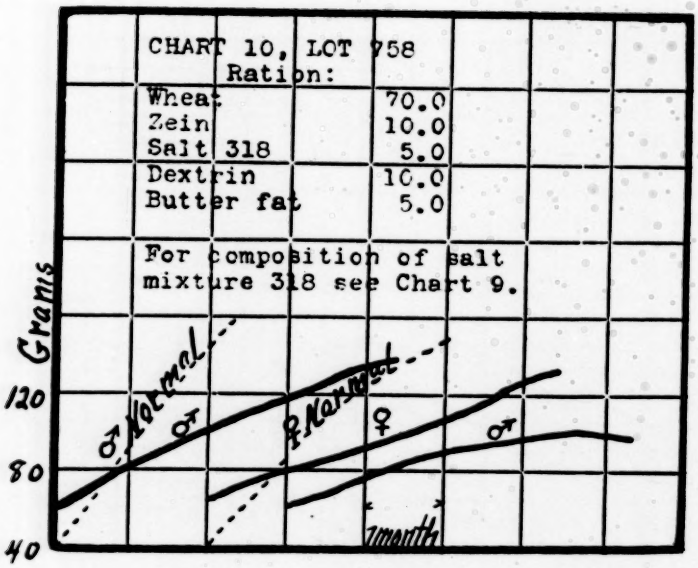


CHART 10. Lot 758 illustrates the failure of zein to supply the limiting amino-acids which determine the value of the wheat proteins. In this ration the addition of more of a protein from any source would lead to better growth, provided the protein added is qualitatively complete (*i.e.*, contains all the essential amino-acids). An *incomplete protein* such as zein will improve the quality of the proteins only when it yields that amino-acid which is present in smallest amount in the proteins of the 70 per cent of wheat in the diet. Since among the indispensable amino-acids zein lacks only lysine and tryptophane, it is apparent that one of these is the limiting factor in the wheat proteins. Osborne and Mendel[1] have shown that for gliadin of wheat lysine is the limiting amino-acid.

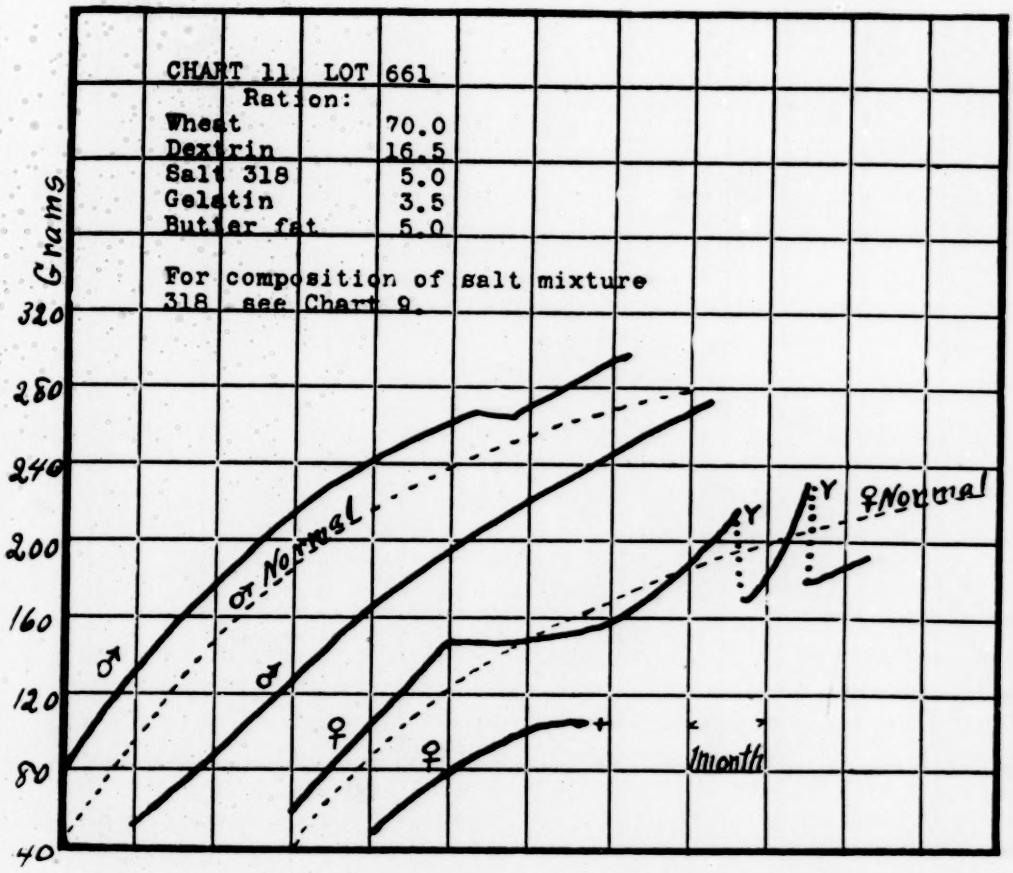


CHART 11. Lot 661 supports the idea that lysine is the limiting amino-acid which determines the value of the protein mixture of the wheat kernel. So small an addition as 3.5 per cent of gelatin, because of its high lysine content, greatly increases the utilizability of the wheat proteins. Without this addition growth takes place at a rate approximating half the normal expectation. With the gelatin addition growth proceeds at the normal rate (compare Charts 9 and 11).

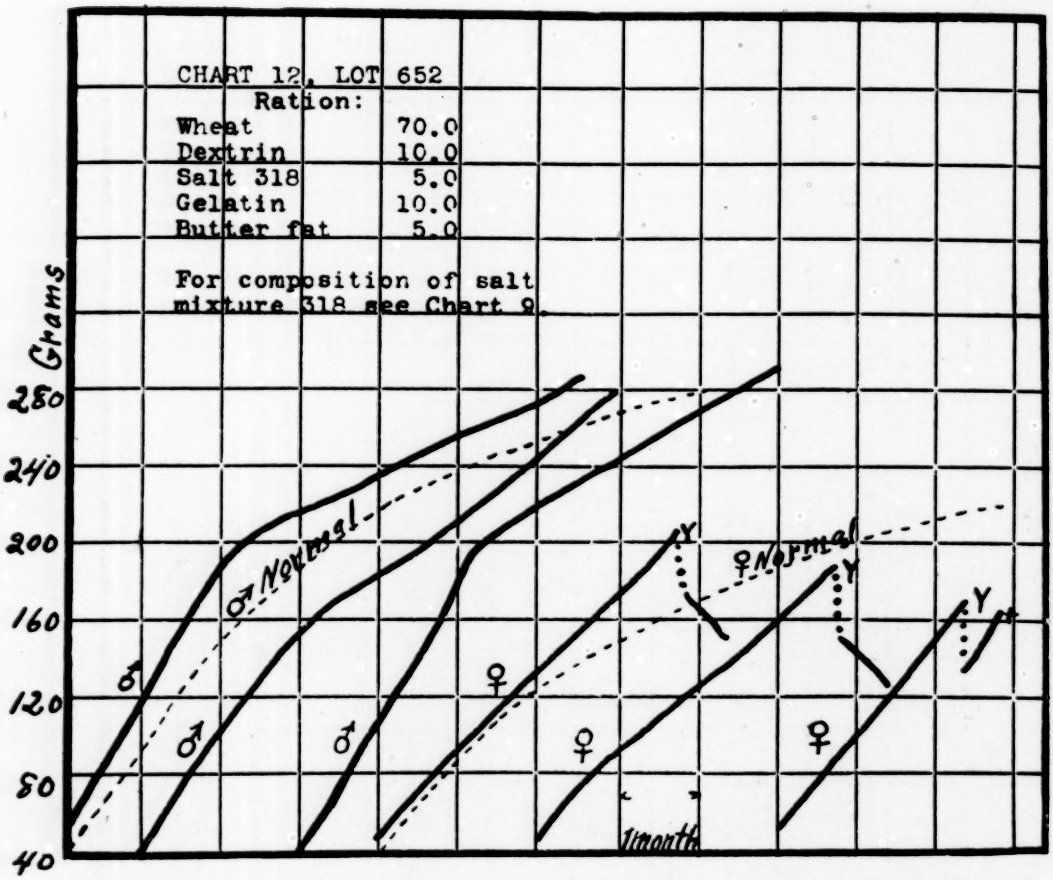


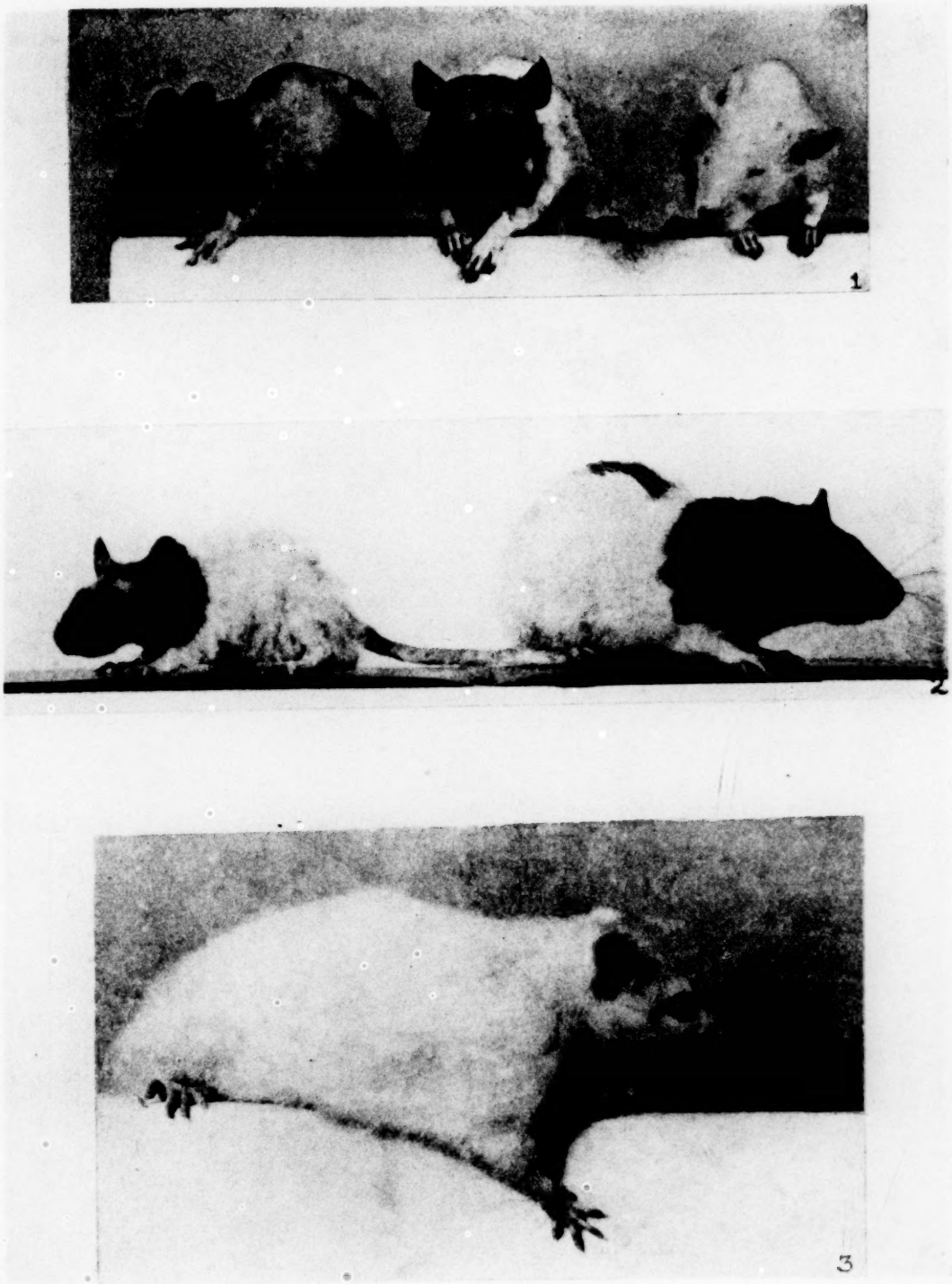
CHART 12. Lot 652 confirms the results obtained with Lot 661 (Chart 11), in showing that gelatin and the protein mixture of the wheat kernel mutually make good the shortage of certain amino-acids in the other and together form a protein mixture which is of much better quality than either component alone. This result is especially interesting when compared with the nutritive value of the proteins of the maize kernel with gelatin added (Charts 1 and 2). The latter combination is no better than the maize protein portion fed alone, and as is well known, gelatin fed as the sole protein is without value as a source of amino-acids. This forms one of the most striking illustrations yet observed of the importance of a knowledge of the supplementary relationship among the proteins of our foodstuffs. Fig. 3 shows the appearance of the rats fed on this mixture.

EXPLANATION OF PLATE 4.

FIG. 1. These animals from Lot 649 (Chart 2) received a diet containing 17 per cent of protein, 10 per cent derived from gelatin and 7 per cent from maize. Gelatin does not improve the proteins of the maize kernel, even though it yields 6 per cent of lysine. This ration with wheat gluten or casein replacing the gelatin induces good growth and well-being. The rats were the same age as those in Fig. 2 when photographed.

FIG. 2. The rations of these two rats were identical except the source of the protein content. The rat on the left received 7 per cent of maize proteins plus 10 per cent of gelatin (Lot 649, Chart 2). The one on the right, from Lot 650 (Chart 4), received 7 per cent of maize proteins plus 10 per cent of wheat gluten protein. Gelatin supplements both wheat and oat proteins, but not those from maize. Both rats were the same age when photographed.

FIG. 3. This rat from Lot 652 (Chart 12) illustrates the good state of nutrition of animals fed wheat supplemented with gelatin. Wheat proteins are greatly improved by being combined with gelatin, whereas maize proteins are not (compare Charts 1 and 2 with 12). This rat is the same age as those in Figs. 1 and 2.



(McCollum, Simmonds, and Pitz: Lysine.)

THE EFFICIENCY OF CERTAIN MILK SUBSTITUTES IN CALF FEEDING.*

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(*From the Laboratory of the Purdue Experiment Station, La Fayette, Ind.*)

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On account of the scarcity of skim milk for calf feeding an attempt was made to find some suitable substitutes. Experiments were carried on with a mixture of various vegetable proteins and also with a mixture of vegetable and animal proteins from various sources. The objects were, first, to determine to what extent a calf meal made up of both animal and vegetable feeding materials, rich in protein, could take the place of skim milk, and, second, to determine whether the proteins from wholly vegetable sources are capable of producing growth and development of the calf to the same extent as the proteins from animal sources.

The feeds were all mixed at the Purdue Dairy Barn, enough of each meal being mixed before the beginning of a period to carry through that period, which insured a uniform meal. A sample was taken from each meal at the time of mixing, for chemical estimation of the nitrogen content, and from this the amount of nitrogen consumed daily was calculated.

The skim milk which was fed was prepared by use of a cream separator and was fed fresh, at a temperature of about 98° F., or practically body temperature. The calf meals were mixed with water, 4 ounces of meal to 3 pounds of water at first, increasing to 12 ounces of meal with 9 pounds of water, and fed at the same temperature as the skim milk. The dry mash, consisting of

* Acknowledgments are due to Professor O. F. Hunziker, Chief of the Dairy Department, for permission to carry on this investigation.

equal parts of ground corn and oats, was fed dry, the amount being carefully weighed to each calf, as was also the alfalfa hay. Salt and water were allowed *ad libitum*.

The calves were kept on as heavy a ration as possible, and were allowed all of each feed that they would readily consume. Whenever it was shown by portions of any feed left unconsumed that the amount given was too great, the rations were reduced accordingly. The care with which the calves were fed and the attending success can be shown by stating that only at two different times during the test was any calf off feed, and that only during one period of 3 to 5 days, in the case of two of these calves, was there a loss of body weight. In each case this loss was less than 1 pound each day during the time.

Feed refused at any time was weighed and a record made of the kind and amount.

FEED CHART.

Feeding Periods and Feeds Received by Each Calf.

Calf.....	B 40	B 43	B 41	B 44
Period I. 29 days.	Skim milk. Alfalfa hay. Dry mash.	Skim milk. Alfalfa hay. Dry mash.	Vegetable meal. Alfalfa hay. Dry mash.	Home mixed meal. Alfalfa hay. Dry mash.
Period II. 25 days.	Home mixed meal. Alfalfa hay. Dry mash.	Vegetable meal. Alfalfa hay. Dry mash.	Skim milk. Alfalfa hay. Dry mash.	Skim milk. Alfalfa hay. Dry mash.
Period III. 18 days.	Vegetable dried blood meal. Alfalfa hay. Dry mash.	Home mixed casein meal. Alfalfa hay. Dry mash.	Vegetable dried blood meal. Alfalfa hay. Dry mash.	Home mixed casein meal. Alfalfa hay. Dry mash.

The meals used were mixed as follows:

1. Vegetable meal.
   Linseed meal.*
   Soy bean meal.
   Cottonseed meal.
   Wheat middlings.
   } Equal parts by weight. N 4.97 per cent.
2. Home mixed meal.
   Hominy feed.
   Linseed meal.*
   White Swan flour.
   Dried blood.
   } Equal parts by weight. N 5.60 " "
3. Vegetable dried blood meal.
   Soy bean meal.
   Linseed meal.*
   Cottonseed meal.
   Wheat middlings.
   Dried blood.
   } Equal parts by weight. N 6.00 " "
4. Home mixed casein meal.

Hominy feed	9 parts by weight.	
Linseed meal.*	9 " " "	N 5.02 " "
White Swan flour	9 " " "	
Casein	8 " " "	

Nitrogen content of the other feeds:

Skim milk	N 0.55 " "
Mash	N 1.46 " "
Alfalfa hay	N 2.53 " "

* Old process.

Average of Periods I, II, and III by Calves.

The figures in the tables are obtained by taking an average of the figures by 3 day periods. These give the average daily nitrogen intake, nitrogen retained, total nitrogen excreted in urine, nitrogen excreted in the form of urea, nitrogen excreted in the feces, the body weight, the body gain, the weight of the urine, and the amount of dry matter or solids in the feces, each in gm., extending over a period of 24 hours.

Tables I and II give detailed illustrative figures selected for two of the four calves used in the feeding experiment.

Figs. 1 to 5 show the amount of nitrogen consumed and its distribution.

TABLE I.

Average Daily Figures by 3 Day Periods.

Date.	Total nitrogen.						Weight of urine.	Dry matter in feces.	Body weight.	Gain.
	Intake.	In urine.	In feces.	Total excreted.	Retained.	Excreted as urea.				
B 41. Period II. Skim Milk Ration.										
1916	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	*kg.*
Jan. 30	63.95	26.79	22.82	49.61	14.34	19.36	1,945*	714	78.9	1.63
" 31–Feb. 2	63.87	20.67	22.54	43.21	20.65	15.17	1,673	712	80.0	1.08
Feb. 3– 5	63.31	19.95	17.62	37.58	25.73	15.18	1,676	568	81.6	1.63
" 6– 8	70.43	20.21	16.14	36.35	34.04	15.75	1,530	548	83.3	1.63
" 9–11	78.37	20.19	22.20	42.39	35.97	15.78	1,500	702	85.6	2.35
" 12–14	81.38	23.43	23.97	47.40	33.98	17.49	1,420	717	89.5	3.81
" 15–17	78.37	25.43	26.79	52.22	26.14	19.25	1,977	794	92.5	3.08
" 18–20	77.96	24.13	28.56	52.69	25.27	17.90	1,688	980	95.1	2.57
" 21–23	74.80	31.39	23.08	54.48	20.32	25.18	2,141	716	96.6	1.51
B 41. Period III. Vegetable Dried Blood Meal Ration.										
Feb. 24–26	83.93	29.86	33.77	63.63	20.29	24.83	1,763	807	97.7	1.08
" 27–29	94.74	25.71	45.19	70.91	23.83	19.69	2,161	1,038	98.3	0.54
Mar. 1– 3	84.36	20.81	47.23	68.05	16.31	15.34	2,348	1,049	101.4	3.08
" 4– 6	96.55	23.90	45.32	69.22	27.32	18.19	1,971	912	105.5	4.17
" 7– 9	96.55	26.94	48.90	75.85	20.69	22.99	2,760	1,040	108.4	2.90
" 10–12	96.55	26.33	47.36	73.70	22.84	21.99	2,316	1,032	111.4	2.94
B 41. Period I. Vegetable Meal Ration.										
Jan. 1– 2	44.78		19.50					471	60.1	
" 3– 5	56.10	14.90	21.15	36.05	20.04	10.68	1,192	540	61.2	1.13
" 6– 8	54.11	18.10	21.54	39.65	14.46	15.34	1,662	488	62.6	1.36
" 9–11	58.14	14.82	24.32	29.15	18.99	12.00	1,285	581	64.0	1.45
" 12–14	61.99	11.11	29.01	40.12	21.86	8.36	1,177	721	66.1	2.08
" 15–17	59.13	19.13	27.47	46.60	12.52	14.67	1,723	658	68.3	2.17
" 18–20	63.09	19.34	31.27	50.61	12.48	13.79	1,629	778	70.5	2.17
" 21–23	64.97	19.28	25.23	44.52	20.45	14.61	2,238	697	72.7	2.17
" 24–26	65.28	19.93	27.06	46.99	18.29	15.67	2,205	760	74.9	2.20
" 27–29	68.41	20.17	26.64	46.81	21.60	16.70	2,054	761	77.3	2.41

* Gephart, F. C., Du Bois, E. F., and Lusk, G., *J. Biol. Chem.*, 1916, xxvii, 217.

TABLE I—*Concluded.*

Date.	Total nitrogen.						Weight of urine.	Dry matter in feces.	Body weight.	Gain.
	Intake.	In urine.	In feces.	Total excreted	Retained.	Excreted as urea.				
B 44. Period II. Skim Milk Ration.										
1916	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	*kg.*
Jan. 30	60.70	17.72	30.26	47.99	12.71	15.18	2,458	742	71.7	1.79
" 31–Feb. 2	57.78	17.93	22.57	40.50	17.27	12.41	1,618	637	72.5	0.87
Feb. 3– 5	57.58	18.88	12.39	31.28	26.29	14.77	1,976	500	73.9	1.36
" 6– 8	64.70	20.27	16.43	36.71	27.99	16.65	1,938	622	75.0	1.11
" 9–11	72.63	25.14	15.50	40.64	31.99	18.71	1,422	519	76.8	1.81
" 12–14	75.65	25.48	22.15	47.63	28.01	19.72	1,494	828	80.1	3.26
" 15–17	75.65	23.72	26.24	49.96	25.68	19.40	2,176	723	83.2	3.08
" 18–20	75.65	21.96	22.84	44.81	30.83	16.61	1,955	744	86.2	2.96
" 21–23	75.65	24.50	22.94	47.44	28.20	19.66	1,594	625	88.9	2.75
B 44. Period III. Home Mixed Casein Meal Ration.										
Feb. 24–26	80.32	28.55	26.18	54.73	25.58	23.33	1,894	754	91.6	2.69
" 27–29	88.07	23.85	29.94	53.79	34.27	19.47	2,527	973	94.3	2.69
Mar. 1– 3	79.75	28.23	33.74	61.97	17.77	21.85	2,619	834	96.9	2.60
" 4– 6	89.87	25.66	35.65	61.31	28.56	20.50	2,767	875	99.3	2.44
" 7– 9	88.67	27.58	35.23	62.81	25.85	22.75	2,522	1,062	101.8	2.45
" 10–12	89.87	28.25	35.74	63.99	25.87	24.14	2,990	929	104.3	2.54
B 44. Period I. Home Mixed Meal Ration.										
Jan. 1– 2	43.34		22.78					411	52.3	
" 3– 5	55.91	11.67	19.13	30.80	25.10	8.46	974	428	54.2	1.81
" 6– 8	56.55	13.88	25.51	39.40	17.15	10.73	1,515	544	56.0	1.81
" 9–11	56.57	13.44	26.51	39.95	16.62	10.53	2,149	601	57.6	1.57
" 12–14	58.05	11.94	22.11	34.05	23.99	9.43	1,992	509	58.7	1.14
" 15–17	57.09	15.01	27.80	42.82	14.27	10.69	1,269	626	60.4	1.72
" 18–20	60.25	16.62	25.16	41.79	18.52	12.69	1,823	703	63.4	2.99
" 21–23	60.38	15.43	25.14	40.58	19.80	11.44	1,505	590	65.5	2.08
" 24–26	60.90	15.15	28.98	44.14	16.75	11.71	1,956	739	67.3	1.75
" 27–29	65.37	15.72	26.36	42.09	23.27	12.37	2,593	699	69.9	2.61

TABLE II.

Average Daily Figures for All Calves, by Periods.

Calf No.	Nitrogen.					Body weight.	Gain.	Weight of urine.	Dry matter in feces.	Ration.
	Intake.	Retained.	In urine.	In feces.	Excreted as urea.					
	Period I.									
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*kg.*	*kg.*	*gm.*	*gm.*	
B 40	56.13	25.51	15.21	15.39	11.81	68.3	2.09	1,135	453	Skim milk.
B 43	69.43	28.78	20.00	20.56	15.57	80.0	2.72	3,039	609	" "
B 41	61.25	17.85	17.42	25.96	13.54	68.6	1.91	1,685	665	Vegetable meal.
B 44	59.01	19.50	14.32	25.19	10.89	61.4	1.73	1,753	604	Home mixed meal.
	Period II.									
B 40	81.63	25.38	18.79	37.45	14.27	88.1	1.87	2,051	827	Home mixed meal.
B 43	83.67	21.44	26.99	35.32	21.03	100.0	1.78	2,537	878	Vegetable meal.
B 41	73.56	27.76	23.17	22.61	17.71	88.0	2.21	1,701	717	Skim milk.
B 44	69.41	27.03	22.24	20.13	17.24	79.5	2.15	1,575	649	" "
	Period III.									
B 40	91.68	19.89	24.42	47.31	19.46	104.0	2.47	2,143	996	Vegetable dried blood meal.
B 43	91.87	26.88	30.80	34.18	24.98	118.9	3.27	2,379	947	Home mixed casein meal.
B 41	92.11	21.88	25.59	44.63	20.51	103.7	2.45	2,220	980	Vegetable dried blood meal.
B 44	86.09	26.32	27.02	32.74	22.01	98.0	2.57	2,553	905	Home mixed casein meal.

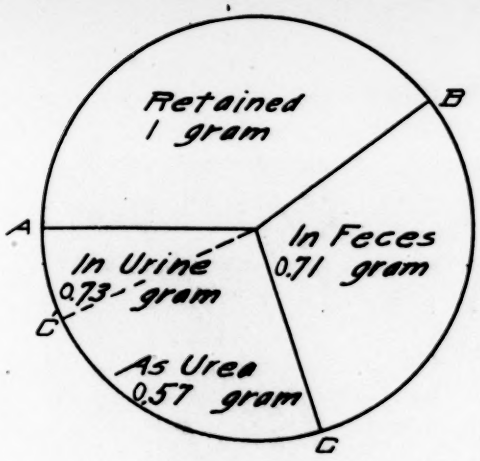


FIG. 1.

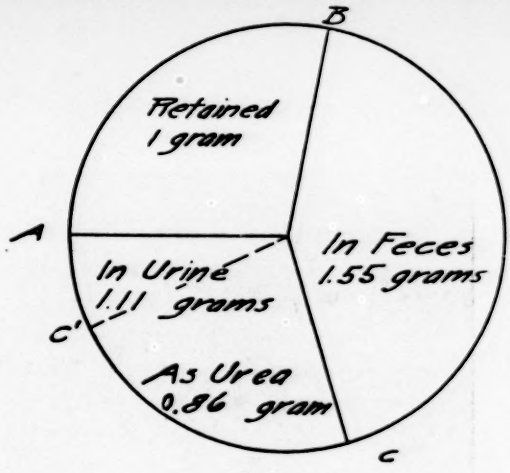


FIG. 2.

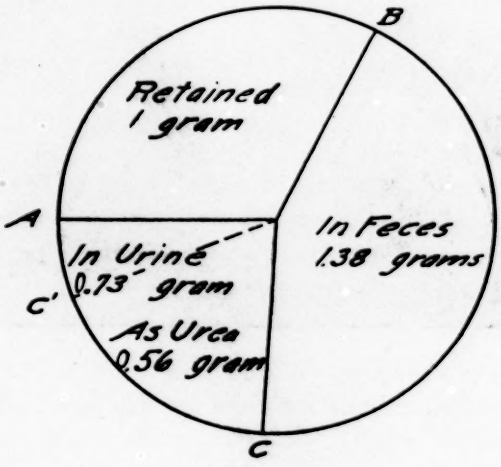


FIG. 3.

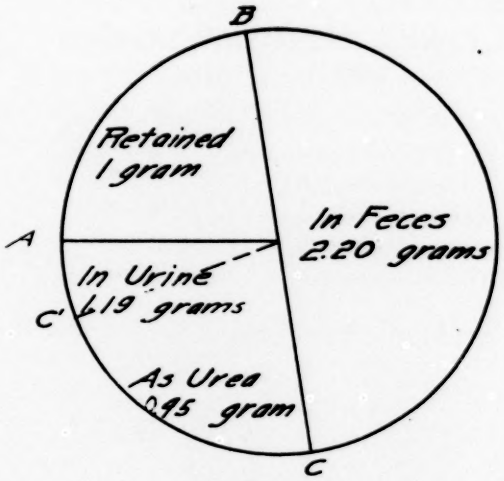


FIG. 4.

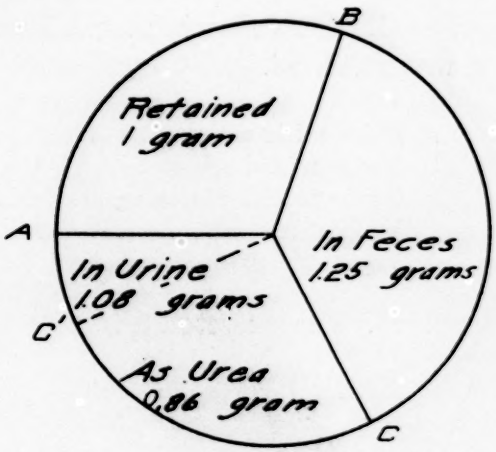


FIG. 5.

FIG. 1. Skim milk ration. Circle = 2.44 gm.
FIG. 2. Vegetable meal ration. Circle = 3.66 gm.
FIG. 3. Home mixed meal ration. Circle = 3.11 gm.
FIG. 4. Vegetable dried blood meal. Circle = 4.39 gm.
FIG. 5. Home mixed casein meal. Circle = 3.33 gm.

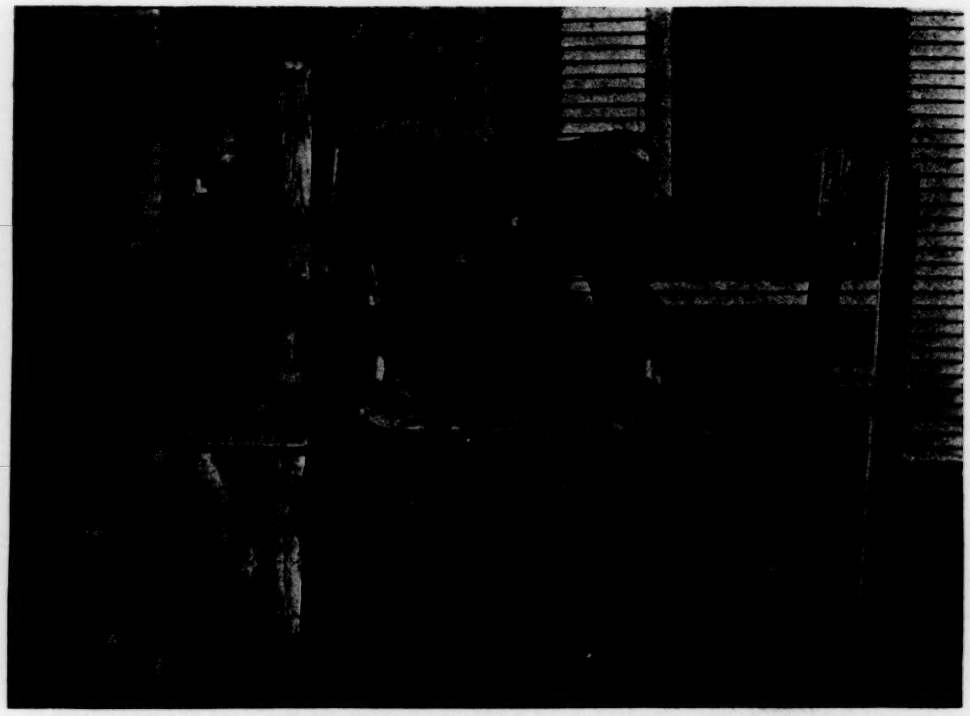


FIG. 6. This crate shows one of the four used in the feeding experiment, and was moved outside of the barn for the purpose of being photographed.

SUMMARY.

I. The following amount of nitrogen consumed in the ration was retained.

	per cent
Skim milk ration	40.7
Home mixed meal	32.0
Home mixed casein meal	30.0
Vegetable meal	27.3
Vegetable dried blood meal	22.6

II. The excreted nitrogen was divided as follows:

	per cent
Skim milk ration: in urine	50.5
" " " " feces	49.5
Home mixed casein meal ration: in urine	46.3
" " " " " " feces	53.7
Vegetable meal ration: in urine	41.2
" " " " feces	58.8

	per cent
Vegetable dried blood meal ration: in urine	35.0
" " " " " " feces	64.9
Home mixed meal ration: in urine	34.2
" " " " " feces	65.2

III. The gains in gm. of body weight per gm. of nitrogen consumed for different rations were:

	gm.
Skim milk	34.41
Home mixed casein meal ration	32.74
Vegetable dried blood meal ration	26.85
Home mixed meal ration	26.17
Vegetable meal ration	26.14

CONCLUSIONS.

1. The nitrogen intake was rather constant per kilo of body weight. The maximum difference was 12 per cent.

2. Less nitrogen was excreted from the vegetable ration than from the dried blood ration, there being a difference of 4.7 per cent.

3. It seems that when the nitrogen in the ration was the most suitable for growth, the nitrogen excreted was about evenly divided between the feces and the urine.

4. The total nitrogen excreted from each of the five rations indicates that the nitrogen in the skim milk ration was absorbed to the greatest advantage and the other feeds in the order named: home mixed meal, home mixed casein meal, vegetable meal, and vegetable dried blood meal.

D-MANNOKETOHEPTOSE, A NEW SUGAR FROM THE AVOCADO.*

By F. B. LA FORGE.

(*From the Carbohydrate Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

(Received for publication, November 31, 1916.)

This article describes the isolation of a new ketose sugar, containing seven carbon atoms, from the fruit of *Persea gratissima*[1] commonly known as avocado or alligator pear. The sugar exists in the free state in the fruit and this fact is noteworthy because there is thus added another monosaccharide to the small number of such substances which have been found to occur in a free state in nature. It is striking that only two monosaccharides, namely, glucose and fructose, have been found widely distributed in any considerable quantity in the free state among all the numerous natural substances that have been investigated; the occurrence of other sugars in nature seems to be usually in combined forms, such as the complex sugars, polysaccharides and glucosides. The new sugar is both a ketose and a heptose and is accordingly the fourth natural ketose to be isolated, the other three being fructose, sorbose, and ketoxylose,[2] and is the first heptose to be found in nature, the previously known heptoses having all been prepared synthetically from hexoses.

The plant in which the new sugar occurs is also the source of the long known *d*-perseite,[3] a heptahydroxy alcohol which has been shown by Fischer[4] to be the alcohol obtained by the

* Published with the permission of the Secretary of Agriculture.

[1] My attention was first called to the presence of an unfermentable sugar in this fruit by Dr. E. E. Butterfield, then of Bellevue Hospital, New York.

[2] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1914, xviii, 319.

[3] Avequin, *Ann. chem. med. Ph. et Toxic*, 1831, vii, 464. Maquenne, *Compt. rend. Acad.*, 1888, cvii, 583.

[4] Fischer, E., and Passmore, F., *Ber. chem. Ges.*, 1890, xxiii, 2226.

reduction of α-mannoheptose. The occurrence in the same fruit of these two similar and rare seven carbon members of the sugar group suggests that there may be some biological relationship between them, and this view receives support from the fact that the new heptose can be transformed into *d*-perseite by reduction with sodium amalgam, as will be described later. There is also produced by this reduction a second crystalline alcohol which appears to be identical with *d*-β-mannoheptite which Peirce[5] has lately prepared from *d*-β-mannoheptose. In Peirce's article conclusive proof is advanced that *d*-perseite (*i.e.*, *d*-α-mannoheptite) has the configuration

$$\begin{array}{l} \quad H \quad H \quad OH \; OH \; H \\ CH_2OH \cdot C \cdot C \cdot C \cdot C \cdot C \cdot CH_2OH \\ \quad OH \; OH \; H \quad H \quad OH \end{array} \qquad \text{(I)}$$

and the corresponding *d*-β-mannoheptite the configuration

$$\begin{array}{l} \quad H \quad H \quad OH \; OH \; OH \\ CH_2OH \cdot C \cdot C \cdot C \cdot C \cdot C \cdot CH_2OH \\ \quad OH \; OH \; H \quad H \quad H \end{array} \qquad \text{(II)}$$

While the formation from the new heptose of two alcohols having these space formulas may be taken to indicate that it has the configuration

$$\begin{array}{l} \quad H \quad H \quad OH \; OH \; O \\ CH_2OH \cdot C \cdot C \cdot C \cdot C \cdot C \cdot CH_2OH \\ \quad OH \; OH \; H \quad H \end{array} \qquad \text{(III)}$$

it is to be remembered that the various alcohols of the sugar group are nearly impossible to distinguish by analysis, and in many cases have such similar properties that positive identification of them is a matter of considerable difficulty. It has, however, been possible to prove conclusively by other means that the avocado sugar has the configuration (III).

Proof That the Avocado Sugar Is d-Mannoketoheptose, of Configuration (III).

The *p*-bromophenylhydrazone of the new sugar was found to contain 20.8 per cent bromine, as an average of three closely

[5] Peirce, G., *J. Biol. Chem.*, 1915, xxiii, 327.

agreeing determinations. Since this hydrazone in the case of a hexose would contain 22.9 per cent bromine, in the case of a heptose 21.1 per cent, and in that of an octose 18.9 per cent, it is evident that the sugar is a heptose. This is in agreement with the fact previously mentioned that the reduction of the sugar yields a heptite, namely, perseite. The percentages of nitrogen and carbon in the *p*-bromophenylhydrazone, as recorded in the experimental part, also point to the same conclusion, which is further corroborated by the estimation of these elements in the phenylosazone of the new sugar. Its nitrogen content was 14.4 per cent as an average of several determinations, and the calculated value for a hexose osazone is 15.6, for that of a heptose 14.4, and in the case of an octose only 13.4. The carbon content was 59.2, and the corresponding values for the osazones of a hexose, heptose, and octose are 60.3, 58.8, and 57.4. This phenylosazone was found to melt at 200° and on comparing this value with the melting points of such of the heptose osazones as are known, it seemed probable that the substance might be identical with *d*-mannoheptose osazone, which Fischer[4] found to melt at about 200°. However, *l*-mannoheptose osazone would of course melt at the same temperature. Since the osazones of the *d* and *l* forms of mannoheptose must have rotations of equal magnitude but opposite sign, a measurement of this property can serve to distinguish the two forms and can also serve as additional evidence that the osazone of one of them is identical with that of the avocado sugar. The osazone of *d*-mannoheptose was prepared and found to rotate in the same direction (right) as the osazone of the avocado sugar, and solutions of the two substances, of equal strengths, in a mixture of pyridine and alcohol, gave the respective values, 0.35 and 0.48°, which do not differ beyond the limits of error. Dr. F. E. Wright in the following article has compared crystals of the two osazones by petrographic-microscopic methods, and finds the substances to be identical. It is, therefore, accepted that the osazone of the avocado heptose is identical with that from *d*-mannoheptose.

According to considerations of structure, *d*-mannoheptose osazone can be derived from three heptoses just as glucose osazone can be prepared from three hexoses, glucose, mannose, and fructose. One of these heptoses is, of course, *d*-α-mannoheptose which Peirce[5] has shown to have the configuration

$$\begin{array}{l} \quad H \quad H \quad OH \; OH \; H \\ CH_2OH \cdot C \cdot C \cdot C \cdot C \cdot C \cdot COH \\ \quad OH \; OH \; H \quad H \quad OH \end{array} \qquad \text{(IV)}$$

A second would be *d*-β-mannoheptose, of configuration

$$\begin{array}{l} \quad H \quad H \quad OH \; OH \; OH \\ CH_2OH \cdot C \cdot C \cdot C \cdot C \cdot C \cdot COH \\ \quad OH \; OH \; H \quad H \quad H \end{array} \qquad \text{(V)}$$

and the third would be a ketose having the configuration already given as (III). To distinguish among these three possible configurations for the avocado heptose, it was noted in the first place that its *p*-bromophenylhydrazone was entirely different in melting point and solubility from the *p*-bromophenylhydrazone of *d*-α-mannoheptose, a fact which excludes configuration (IV). Regarding *d*-β-mannoheptose, configuration (V), Peirce records that he was unable to crystallize its *p*-bromophenylhydrazone, whereas the avocado heptose yielded this derivative readily. Since configuration (V) is thus excluded, the new sugar can only be represented by the ketose formula (III), and must accordingly be named *d-mannoketoheptose.*

Further proof of its ketose nature was obtained from the fact that bromine in aqueous solution was without action upon it, corresponding to the general observation[6] that bromine oxidizes aldoses readily but does not affect ketoses.

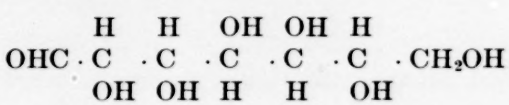
Considerations on the Configuration of Perseulose.

Bertrand[7] has obtained from *d*-perseite, through the action of *Bacterium xylinum*, a seven carbon sugar, perseulose, which he records as having the following properties: melting point 110–115°, $[\alpha]_D$ initial value −90 mutarotating to −80°, and yielding an osazone of melting point 230°. These properties are sufficient to differentiate it from the avocado sugar, nor can it be identical with either of the mannoaldoheptoses which yield the same osazone as the avocado sugar. While these data are sufficient to prove that the avocado sugar is not perseulose and that perseulose

[6] Votoček, E., and Němeček, J., *Z. Zuckerind. Böhmen*, 1910, xxxiv, 399. Nef, J. U., *Ann. Chem.*, 1914, cdiii, 204.

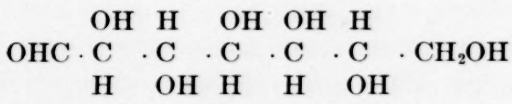
[7] Bertrand, G., *Compt. rend. Acad.*, 1908, cxlvii, 201; 1909, cxlix, 225.

is not one of the mannoaldoheptoses, they do not show positively the configuration of perseulose. It appears, however, from the following additional evidence that its configuration can be established with a fair degree of certainty. Referring back to the configuration of *d*-perseite (I) from which perseulose is derived, it has just been proved that the oxidation to the sugar does not take place at the carbon atom at the extreme right in the formula nor at the carbon atom in the α position to it, because perseulose osazone is not identical with that from the sugars having the structures (III), (IV), and (V), as mentioned above. If it should be that the oxidation takes place at the carbon atom at the extreme left or at the carbon atom in the α position to it, a sugar would be obtained which should yield an osazone identical with that from the two *l*-galaheptoses



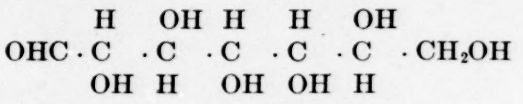
(VI)

and



(VII)

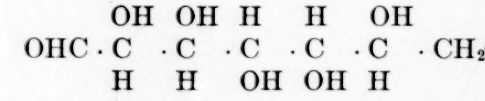
The melting point of perseulose osazone was found to be 230° by Bertrand, and Fischer[8] records the melting point of *d*-galaheptose osazone as 224°, and the melting point of *l*-galaheptose osazone would of course be the same. I have prepared the osazone of *d*-galaheptose and found it to melt at 222°, which agrees with Fischer's determination and is sufficiently close to the value found by Bertrand, when it is remembered that the melting points of the osazones depend considerably on the rate of heating, to indicate with a fair degree of certainty that perseulose osazone is identical either with *d*- or *l*-galaheptose osazone. Peirce has proved that crystalline *d*-β-galaheptose has the configuration



(VIII)

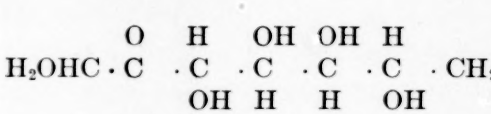
[8] Fischer, *Ann. Chem.*, 1895, cclxxxviii, 139.

which is the antipode of (VII). Neither (VII) nor (VIII) can be the configuration of perseulose because neither can give the configuration of *d*-perseite by reduction. The other *d*-β-galaheptose of configuration



(IX)

the antipode of (VI), was obtained by Fischer as an uncrystallizable sirup, whereas perseulose crystallizes readily. Hence, perseulose cannot be identical with either of these sugars and there remains the configuration



(X)

This conclusion is supported by Bertrand's generalization that the oxidation of the alcohols of the sugar group by *Bacterium xylinum* only takes place at the carbon atom in an α position, and only then in case the hydroxyl on the β carbon atom is on the same side of the configuration as that on the α carbon.

The conclusion that configuration (X) applies to perseulose requires that perseulose osazone be identical with *l*-galaheptose osazone, rather than with that from *d*-galaheptose, a deduction which is not obtainable alone from the identity of the melting points of the osazones. The osazone of *d*-galaheptose which melted at 222°, as mentioned, was found to rotate to the right. Hence, it must be concluded that *l*-galaheptose osazone, which seems to be identical with perseulose osazone, rotates to the left. The osazone of the avocado sugar rotates to the right, thus completing the evidence that the sugar itself cannot be identical with perseulose or with either of the *l*-galaheptoses.

In addition to *d*-mannoketoheptose and *d*-perseite the pulp of the avocado yielded another carbohydrate; namely, a gum insoluble in aqueous alcohol which gave on hydrolysis *l*-arabinose. The pentose was isolated in the form of its benzylphenylhydrazone.

It should be mentioned that no starch was found in the ripe fruit of the avocado.

In the following article are given crystallographic and optical measurements of mannoketoheptose and an optical comparison of the osazones of mannoketoheptose and mannoaldoheptose.[9]

EXPERIMENTAL.

Preparation of d-Mannoketoheptose from the Avocado.

The pulp of twelve avocados (Trapp variety), the aggregate weight of which was 3,500 gm., was passed through a sieve, mixed with about 6 liters of water, and filtered over night on folded filters. The residue was again extracted with the same amount of water and the combined filtrates were concentrated in a large dish on the steam bath to about 4 liters. The separated protein was removed by filtration and the solution concentrated under diminished pressure, with the addition of amyl alcohol to prevent foaming, to about 1.5 liters. 6 liters of about 98 per cent alcohol were added and the separated gum was filtered off with suction. The filtrate was then concentrated under diminished pressure to 250 to 300 cc. and sufficient absolute alcohol added to cause a permanent turbidity. After standing over night in the ice box, the perseite that had crystallized out was filtered off and the alcoholic solution was concentrated under diminished pressure to a thick sirup. It may be stated at this point that the first crystals of the sugar were obtained by preparing from such a sirup the crystalline *p*-bromophenylhydrazone, decomposing it with benzaldehyde and allowing the concentrated solution of the regenerated sugar to crystallize spontaneously. In later preparations it was found simpler to use the following procedure after crystals of the sugar had become available for seeding. The sirup referred to above was mixed with an equal volume of glacial acetic acid,[10] seeded with a few crystals of the sugar, and allowed

[9] These were made by Dr. F. E. Wright of the Geophysical Laboratory of this city, to whom I wish to express my thanks. I also wish to thank Dr. C. S. Hudson for revising the manuscript of this article and for helpful suggestions during the course of the work.

[10] According to the method of A. Wernicke, for crystallizing cane sugar (*Ber. chem. Ges.*, 1882, xv, 3105), and U. S. Patent No. 260,340 of June 27, 1882. Dr. I. K. Phelps was the first to use this method in the Bureau of Chemistry.

to stand 3 or 4 days in a desiccator. The crystals which had formed were filtered from the sirupy mother liquor with suction, washed with glacial acetic acid, and finally with alcohol. The yield was about 50 gm. The sugar was recrystallized by dissolving in a very small amount of water and adding several volumes of absolute alcohol. Often, when solutions of the sugar were allowed to evaporate slowly, crystals of 1 to 2 mm. diameter were formed, which appeared as six-sided prisms. The pure sugar melts at 152° uncorrected, without decomposition. Like other heptoses and like the pentoses, it gives a color reaction with orcin and hydrochloric acid. In aqueous solution it showed a dextrorotation as follows:

$$\text{I.}\quad [\alpha]_D^{20} = \frac{+2.8^\circ \times 5.5243}{1 \times 1.036 \times 0.5083} = +29.37^\circ$$

$$\text{II.}\quad [\alpha]_D^{20} = \frac{+2.76^\circ \times 5.5013}{1 \times 1.034 \times 0.5069} = +28.97^\circ$$

No mutarotation was observed.

0.1445 gm. substance gave 0.2127 gm. CO_2 and 0.0868 gm. H_2O.

	Calculated for $C_7H_{14}O_7$:	Found:
C	40.00	40.14
H	6.66	6.66

It is not fermentable with yeast nor is the aqueous extract of the fruit.

p-Bromophenylhydrazone.—16 gm. of the thick crude sugar sirup, already referred to, were dissolved in 8 cc. of H_2O, and a solution of 12 gm. of *p*-bromophenylhydrazine in 125 cc. of absolute alcohol was added. The mixture was warmed a few minutes on the steam bath until all was dissolved and was then allowed to stand 24 hours at room temperature. The solvents were distilled off under diminished pressure and the resulting light brown sirup was mixed with about two volumes of cold water. Crystallization of the hydrazone began at once and was complete after standing for a few hours in the ice box. After filtering with suction and washing with water and then with ether until colored material was no longer removed, the hydrazone was recrystallized from the least possible amount of hot water. The

product thus obtained was recrystallized from alcohol, of which about the same amount was necessary for solution as was used of water in the first instance. For a final purification it was again recrystallized from absolute alcohol, of which about five times the above amount was necessary for solution of the purified material. The yield was 10 to 12 gm. The hydrazone crystallizes in thin plates which are usually slightly yellow. It melts at 179° uncorrected.

0.1348 gm. substance gave 0.0662 gm. AgBr.
0.2777 " " " 0.1366 " "
0.2390 " " " 0.1166 " "
0.1479 " " " 10.1 cc. N_2 at 767 mm. and 24°.
0.1316 " " " 0.1985 gm. CO_2 and 0.0579 H_2O.

	Calculated for $C_{13}H_{19}N_2BrO_6$:	Found:
C	41.16	41.13
H	5.01	4.88
N	7.39	7.69
Br	21.10	I. 20.89 II. 20.75 III. 20.94

Cleavage of Bromophenylhydrazone.—5.75 gm. of hydrazone were suspended in 250 cc. of 30 per cent alcohol, 4.5 gm. of benzaldehyde added, and the solution was boiled over a free flame until the alcohol was expelled. The *p*-bromophenylhydrazone of benzaldehyde separated out oily at first, but crystallized on cooling. This was filtered off and the filtrate extracted repeatedly with ether and concentrated to a thick sirup under diminished pressure. This was stirred up with very little absolute alcohol and allowed to stand in a desiccator. Crystallization took place after about a week. The yield of the sugar was almost quantitative.

The Phenylosazone of d-Mannoketoheptose.—This was prepared in the usual way from a 1 per cent solution of the pure sugar. It was recrystallized from dilute alcohol and washed with absolute alcohol. It melted at about 200° with rapid heating and the substance mixed with the osazone of mannoaldoheptose melted simultaneously with the separate samples.

0.1375 gm. substance gave 0.2987 gm. CO_2 and 0.0771 H_2O.
0.1485 " " " 19.9 cc. N_2 at 756 mm. and 18°.
0.1457 " " " 18.4 " " " 768 " " 22°.

	Calculated for $C_{19}H_{34}O_6N_2$:	Found:
C	58.76	59.24
H	6.19	6.23
N	14.43	I. 14.55 II. 14.38

A solution of 0.1000 gm. of osazone from the avocado sugar in 5 cc. of pyridine alcohol mixture rotated with sodium light in a 0.5 dm. tube after about 15 minutes + 0.74°, to the right, and after 24 hours + 0.35°.

A solution of 0.1000 gm. of osazone from mannoaldoheptose under the same conditions rotated after 15 minutes + 0.78°, to the right, and after 24 hours + 0.48°.

Attempt to Oxidize the Avocado Sugar with Bromine.—An aqueous solution of the sugar, 5 cc. of which corresponded to (I) 0.0964 gm. of Cu_2O, (II) 0.0954 gm. of Cu_2O, was allowed to stand for 4 days with an excess of bromine. After this time 5 cc. were boiled to remove the bromine and corresponded to 0.0952 gm. of Cu_2O, which shows that no appreciable oxidation had taken place.

Reduction of Heptose.—6.5 gm. of the avocado sugar were reduced with sodium amalgam in the usual way. The solution was kept at about 0° for the first 4 hours and neutralized with sulfuric acid frequently throughout the reaction. After about 8 hours' action of the amalgam, the solution which then showed no reduction with Fehling's solution was concentrated under diminished pressure until a large part of the sodium sulfate had crystallized out. It was then poured into 1.5 liters of 95 per cent alcohol and the alcoholic solution concentrated to about 15 cc. and diluted with about 200 cc. of absolute methyl alcohol. On standing 24 hours, 1.25 gm. of *d*-perseite crystallized out. This was once recrystallized from dilute methyl alcohol and it melted then at 188°. It rotated in saturated aqueous borax solution as follows:

$$[\alpha]_D^{20} = \frac{+0.41° \times 5.6062}{1 \times 0.5069} = +4.53°$$

The characteristic heptacetate of *d*-perseite was obtained by warming one part of the crystals with ten parts of acetic anhydride in the presence of a trace of zinc chloride. The reaction mixture was poured into water and after the oil which first separated out had crystallized, the product was filtered off and purified by recrystallization from 70 per cent alcohol. It melted at 119°.

0.1526 gm. substance gave 0.0831 gm. H_2O and 0.2806 gm. CO_2.

	Calculated for $C_{21}H_{30}O_{14}$:	Found:
C	49.80	50.14
H	5.92	6.05

From the filtrate from perseite the second heptite was obtained on evaporation as a sirup which crystallized on slow addition of alcohol and stirring. After washing with 95 per cent alcohol and drying, the yield amounted to 2.6 gm. This second hexite was recrystallized three times from fifteen to twenty parts of 95 per cent alcohol, and it melted at about 140° to a turbid viscous liquid which did not become clear until the temperature was raised to about 215°. It was, therefore, sublimed in a high vacuum and was then found to melt simultaneously with a sample of *d*-β-mannoheptite prepared by Peirce, at 149–155°, uncorrected.

0.1425 gm. substance gave 0.0952 gm. H_2O and 0.2071 gm. CO_2.

	Calculated for $C_7H_{16}O_7$:	Found:
C	39.60	39.63
H	7.64	7.42

In aqueous solution it showed a dextrorotation as follows:

$$[\alpha]_D^{20} = \frac{+0.24° \times 5.4987}{1 \times 0.5174} = +2.55°$$

0.4994 gm. in 5 cc. of saturated aqueous borax solution rotated in a 1 dm. tube + 0.93°.

d-Galaheptose Osazone.—This was prepared in the usual way from *d*-α-galaheptose.[8] It crystallized from methyl alcohol in long needles which melted at 216° (222° corrected). A solution of 0.1 gm. of substance in 10 cc. of pyridine alcohol mixture

rotated in a 1 dm. tube and D-light + 0.60°, to the right, and after 48 hours + 0.40°.

p-Bromophenylhydrazone of d-Mannoaldoheptose.—Equal parts of α-mannoaldoheptose and *p*-bromophenylhydrazine were dissolved on the steam bath in thirty parts of 75 per cent alcohol, and the solution was allowed to stand for 24 hours at room temperature. The yield was quantitative. The substance is difficultly soluble in water. It was recrystallized from a large volume of alcohol, in which it is also extremely difficultly soluble. It melted at 207–208° uncorrected.

0.2020 gm. substance gave 13.60 cc. N_2 at 768 mm. and 25°.

	Calculated for $C_{13}H_{19}N_2BrO_6$:	Found:
N	7.39	7.55

Hydrolysis of the Gum from the Avocado.—100 gm. of gum which had been purified by dissolving in a small amount of water, filtering, and precipitating with alcohol were hydrolyzed for 3 hours with 1 liter of about 9 per cent sulfuric acid. The acid was removed with barium carbonate and the filtrate concentrated to a sirup which was extracted with hot alcohol. The alcoholic solution was concentrated under diminished pressure to a small volume and the benzylphenylhydrazone of *l*-arabinose prepared by adding to the warm solution the calculated amount of benzylphenylhydrazine. After recrystallization from alcohol, it melted at 174° uncorrected.

0.2044 gm. substance gave 15.8 cc. N_2 at 772 mm. and 25°.

	Calculated for $C_{18}H_{22}N_2O_4$:	Found:
N	8.48	8.72

l-Arabinose Osazone.—This derivative was prepared from the hydrolytic product from the gum in the usual way. It was recrystallized three times from water and melted then at 158° uncorrected.

0.1000 gm. of substance in 10 cc. of pyridine alcohol mixture rotated in a 1 dm. tube with sodium light after 10 minutes, + 0.60°, to the right, and after 6 hours + 0.30° in the same direction.

CRYSTALLOGRAPHIC AND OPTIC PROPERTIES OF MANNOKETOHEPTOSE AND OF THE OSAZONES OF MANNOKETOHEPTOSE AND MANNO-ALDOHEPTOSE.

By F. E. WRIGHT.

(*From the Geophysical Laboratory, Washington.*)

(Received for publication, November 31, 1916.)

The crystals which served for the measurements recorded below were selected from a preparation kindly furnished to me by Dr. La Forge. These crystals are transparent, pale yellow in color, and thick tabular to equant in habit. No evidence of good cleavage was observed. The fracture is conchoidal. The crystals average about 2 mm. in diameter and are bounded on all sides by crystal faces, many of which are, however, imperfect and give multiple reflection signals and light streaks so that crystallographic measurements of a high degree of precision cannot be made. The crystals were measured on a Goldschmidt two-circled goniometer with reducing attachment. The average results of the measurements of five crystals are listed in Table I;

TABLE I.

Mannoketoheptose. Monoclinic Sphenoidal.

$a : b : c = 1.458 : 1 : 0.921$. $\beta = 116° 36'$.

$p_0 = 0.631$; $q_0 = 0.823$; $e = 0.448$; $h = 0.894$; $a_0 = 1.584$; $b_0 = 1.086$.

No.	Letter.	Goldschmidt.	Miller.	Computed.		Observed.	
				φ	ρ	φ	ρ
1	c	0	001	90° 00′	26° 36′	90° 00′	26° 36′
2	a	∞ 0	100	90 00	90 00	90 06	90 00
3	m	∞	110	37 29	90 00	38 59	90 00
4	m′	∞ $\bar{\infty}$	1$\bar{1}$0	142 31	90 00	142 31	89 54
5	r	$\bar{1}$0	$\bar{1}$01	−90 00	11 35	−90 00	11 57
6	s	$\bar{2}$0	$\bar{2}$01	−90 00	42 20	−89 32	42 46
7	d	1	111	52 40	56 37	58 30	50 59

also the results obtained by computation from the weighted measurements of the angles: 001: $\bar{1}01$ = 38°33′; 100: $1\bar{1}0$ = 52° 31′; 001: 100 = 63°24′.

The forms 001, 100, 110, $1\bar{1}0$, $\bar{1}01$ were present on all five crystals; the forms 111, $\bar{2}01$ were observed only once; the face 111 was so rounded and poorly developed that it cannot be considered to be an established form. The faces 110, $\bar{1}10$ were invariably rounded and gave, on the goniometer, light streak signals whose general directions were in accord with the monoclinic sphenoidal symmetry of the crystal; the faces $1\bar{1}0$, $\bar{1}\bar{1}0$, on the other hand, were commonly flat and gave single, sharp re-

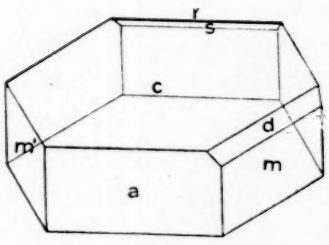


FIG. 1.

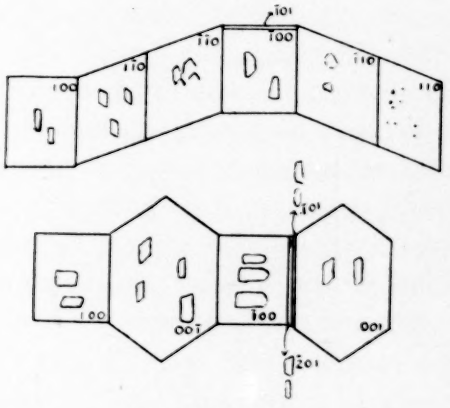


FIG. 2.

FIG. 1. Crystal of mannoketoheptose.
FIG. 2. Etch figures on different faces of a crystal of mannoketoheptose.

flection signals. This pronounced difference in the character of development of the prism faces on opposite sides of the plane of symmetry indicates that there is a digonal polar axis of symmetry, but not a plane of symmetry, present; this would place the crystal in the monoclinic hemimorphic class (digonal polar type) with the *b*-axis as the digonal axis of symmetry.

To test this inference a number of the crystals were immersed for 10 seconds in 95 per cent alcohol and etch figures produced. The etch figures were small and unequally developed on the different faces; they were studied both in reflected and in transmitted light. Some of the more commonly observed shapes are indicated in Fig. 2. These prove that there is no plane of sym-

metry present; also that the flat prism faces $1\bar{1}0$, $\bar{1}\bar{1}0$ react, with respect to the solvent, differently from the faces 110, $\bar{1}10$. These relations suffice to establish the polar character of prism faces.

The optical relations are in accord with the monoclinic symmetry deduced from the crystallographic data. The optical orientation is: $b = \beta$; $c: \gamma = 26°36'$. Optic axial angle $2E > 180°$; optical character +. The refractive indices were measured in sodium light with an Abbe-Pulfrich total refractometer on the flat basal pinacoid of a crystal about 1 sq. mm. in area. $\alpha = 1.547$, $\beta = 1.570$, $\gamma = 1.595$; $\gamma - \alpha = 0.048$, $\gamma - \beta = 0.025$, $\beta - \alpha = 0.023$. The acute bisectrix γ is normal to the basal pinacoid; this was proved by direct observation of the interference figure from the basal pinacoid; also by measurement of the optic axial angle on the axial angle apparatus; and by the straight boundary line of the refractive index γ as observed on the total refractometer during a complete rotation of the crystal about the vertical axis.

The optic axial angle was measured directly on a small crystal immersed in a refractive liquid of index $\beta = 1.570$; an average value of $2V = 89°$ was obtained. No appreciable dispersion was noted either of the bisectrices or of the optic axes. One of the optic axes on each side of the crystal presented an abnormal appearance as though there were two optic axes, the one directly under the first; the zero isogyre of this optic axis was inclined at an appreciable angle with the principal Nicol planes. The phenomena were, however, indistinct because of the smallness of the crystal, and this interesting aspect of the optic axis was not investigated further. The optic axial angle was also computed from the refractive indices and the value $2V = 89°$ obtained; the excellent agreement between this computed value and the above measured value is the result rather of chance than of precision in the measurements. It is significant, however, that in both cases γ is found to be the acute bisectrix; the crystals are, therefore, evidently optically positive.

The Osazones of Mannoketoheptose and Mannoaldoheptose.

The two preparations consist, as far as can be determined, of the same crystal substance. They are lemon-yellow powders, very fine-grained, and more or less aggregated in loose clusters.

Under the microscope one of the samples is seen to consist of fine radial spherulites averaging 0.04 mm. in diameter. The second is composed chiefly of fine needles 0.01 to 0.02 mm. long and 0.002 to 0.005 mm. wide; in it fan-shaped portions of radial spherulites are also present in small amounts. The needles of both samples are strongly refracting, the average refractive index being approximately 1.775. The birefringence is medium. Especially characteristic are the abnormal interference colors which range from orange-yellow to blue-green and are the result of remarkably large dispersion of the bisectrices and also of strong spectral absorption of all light waves below 470 $\mu\mu$. A measurement of the amount of dispersion was made with the aid of a monochromatic illuminator. The extinction angle on one of the needles varied with the wave length as follows:

Wave length in $\mu\mu$:	616	577	552	514	495
Extinction angle (c : α') :	15°	25.5°	34°	44°	50°

Below 490 $\mu\mu$ the crystals absorb the light so strongly that at 470 $\mu\mu$ practically no light is transmitted even in the thinnest needles. This fact was tested further by observing that the needles illuminated by the blue and violet lines (435, 407, 404 $\mu\mu$) of an intense quartz mercury arc appeared perfectly black and opaque.

As a result of the strong dispersion of the bisectrices the needles do not show in white light a position of total extinction but only a rapid change in hue from orange-yellow to blue-green at the position of normal extinction. The elongation of the fibers is positive (γ').

These optical data suffice to prove that the crystal system of this substance is either monoclinic or triclinic.

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